cyte infiltration can interrupt the positive feedback cycle between inflammation and atherogenesis.

We previously reported that CCR2 expression and function are enhanced in circulating monocytes in hypertensive animals and humans through an AT1 receptor-mediated mechanism; increased CCR2 on monocytes is an important predictor of the presence of hypertension; and monocyte CCR2 is critical for monocyte-mediated inflammation and remodeling in Ang II-induced hypertension in mice.[21] Our present data extend our previous study by showing the decisive role of CCR2 on monocyte in Ang II-induced acceleration of atherosclerosis. Overall, these findings rule out the possibility that expression of CCR2 by resident arterial cells is involved primarily in the mechanism of Ang II-induced vascular remodeling and atherosclerosis. However, Sata[22] and other investigators have reported that BM-derived progenitor cells can migrate to atherosclerotic lesions, differentiate into vascular wall cells, and thus contribute to the development of vascular remodeling and atherosclerosis. Although the degrees to which BM-derived progenitors contribute to the mechanism of vascular disease remain unclear, we do not completely exclude the possibility that expression of CCR2 by BM-derived progenitor cells other than circulating monocytes contributed to the present results.

In conclusion, the present study provides first evidence that CCR2 expressed on monocytes has a critical role in Ang II-induced acceleration of the atherosclerotic process. This finding might also apply to the vascular pathology of atherosclerosis caused by other stimuli such as hypercholesterolemia and/or hypertension, because enhanced CCR2 expression on circulating monocytes has been demonstrated in animals and humans with hypercholesterolemia[23] or hypertension.[21]

Acknowledgments

This study was supported by grants-in-aid for Scientific Research (14657172, 14207036) from the Ministry of Education, Culture, Sports, Science, and Technology, Tokyo, Japan; Health Science Research grants (Comprehensive Research on Aging and Health, and Research on Translational Research) from the Ministry of Health, Labor, and Welfare, Tokyo, Japan; and by the Program for Promotion of Fundamental Studies in Health Sciences of the Organization for Pharmaceutical Safety and Research, Tokyo, Japan.

References

1. Libby P. Current concepts of the pathogenesis of the acute coronary syndromes. *Circulation*. 2001;104:365–372.
2. Kolodgie FD, Gold HK, Burke AP, Fowler DR, Kruth HS, Weber DK, Farb A, Guerrero LJ, Hayase M, Kutys R, Narula J, Finn AV, Virmani R. Intraplaque hemorrhage and progression of coronary atheroma. *N Engl J Med*. 2003;349:2316–2325.
3. Dzau VJ. Theodore Cooper Lecture: Tissue angiotensin and pathobiology of vascular disease: a unifying hypothesis. *Hypertension*. 2001;37: 1047–1052.
4. Brasier AR, Recinos A 3rd, Eledrisi MS. Vascular inflammation and the renin-angiotensin system. *Arterioscler Thromb Vasc Biol*. 2002;22: 1257–1266.
5. Griendling KK, Ushio-Fukai M, Lassegue B, Alexander RW. Angiotensin II signaling in vascular smooth muscle. New concepts. *Hypertension*. 1997;29:366–373.
6. Daugherty A, Manning MW, Cassis LA. Angiotensin II promotes atherosclerotic lesions and aneurysms in apolipoprotein E-deficient mice. *J Clin Invest*. 2000;105:1605–1612.
7. Weiss D, Kools JJ, Taylor WR. Angiotensin II-induced hypertension accelerates the development of atherosclerosis in apoE-deficient mice. *Circulation*. 2001;103:448–454.
8. Gerard C, Rollins BJ. Chemokines and disease. *Nat Immunol*. 2001;2: 108–115.
9. Mukaida N, Harada A, Matsushima K. Interleukin-8 (IL-8) and monocyte chemotactic and activating factor (MCAF/MCP-1), chemokines essentially involved in inflammatory and immune reactions. *Cytokine Growth Factor Rev*. 1998;9:9–23.
10. Egashira K. Molecular mechanisms mediating inflammation in vascular disease: special reference to monocyte chemoattractant protein-1. *Hypertension*. 2003;41:834–841.
11. Ni W, Kitamoto S, Ishibashi M, Usui M, Inoue S, Hiasa K, Zhao Q, Nishida K, Takeshita A, Egashira K. Monocyte chemoattractant protein-1 is an essential inflammatory mediator in angiotensin II-induced progression of established atherosclerosis in hypercholesterolemic mice. *Arterioscler Thromb Vasc Biol*. 2004;24:534–539.
12. Boring L, Gosling J, Cleary M, Charo IF. Decreased lesion formation in CCR2−/− mice reveals a role for chemokines in the initiation of atherosclerosis. *Nature*. 1998;394:894–897.
13. Gu L, Okada Y, Clinton SK, Gerard C, Sukhova GK, Libby P, Rollins BJ. Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. *Molecular Cell*. 1998; 2:275–281.
14. Ni W, Egashira K, Kitamoto S, Kataoka C, Koyanagi M, Inoue S, Imaizumi K, Akiyama C, Nishida Ki K, Takeshita A. New anti-monocyte chemoattractant protein-1 gene therapy attenuates atherosclerosis in apolipoprotein E-knockout mice. *Circulation*. 2001;103:2096–2101.
15. Inoue S, Egashira K, Ni W, Kitamoto S, Usui M, Otani K, Ishibashi M, Hiasa K, Nishida K, Takeshita A. Anti-monocyte chemoattractant protein-1 gene therapy limits progression and destabilization of established atherosclerosis in apolipoprotein E-knockout mice. *Circulation*. 2002;106:2700–2706.
16. Egashira K, Zhao Q, Kataoka C, Ohtani K, Usui M, Charo IF, Nishida K, Inoue S, Katoh M, Ichiki T, Takeshita A. Importance of monocyte chemoattractant protein-1 pathway in neointimal hyperplasia after periarterial injury in mice and monkeys. *Circ Res*. 2002;90:1167–1172.
17. Jiang Y, Beller DI, Frendl G, Graves DT. Monocyte chemoattractant protein-1 regulates adhesion molecule expression and cytokine production in human monocytes. *J Immunol*. 1992;148:2423–2428.
18. Salcedo R, Ponce ML, Young HA, Wasserman K, Ward JM, Kleinman HK, Oppenheim JJ, Murphy WJ. Human endothelial cells express CCR2 and respond to MCP-1: direct role of MCP-1 in angiogenesis and tumor progression. *Blood*. 2000;96:34–40.
19. van Royen N, Hoefer I, Bottinger M, Hua J, Grundmann S, Voskuil M, Bode C, Schaper W, Buschmann I, Piek JJ. Local monocyte chemoattractant protein-1 therapy increases collateral artery formation in apolipoprotein E-deficient mice but induces systemic monocytic CD11b expression, neointimal formation, and plaque progression. *Circ Res*. 2003;92:218–225.
20. Viedt C, Vogel J, Athanasiou T, Shen W, Orth SR, Kubler W, Kreuzer J. Monocyte chemoattractant protein-1 induces proliferation and interleukin-6 production in human smooth muscle cells by differential activation of nuclear factor-kappaB and activator protein-1. *Arterioscler Thromb Vasc Biol*. 2002;22:914–920.
21. Ishibashi M, Hiasa K, Zhao Q, Inoue S, Ohtani K, Kitamoto S, Tsuchihashi M, Sugaya T, Charo IF, Kura S, Tsuzuki T, Ishibashi T, Takeshita A, Egashira K. Critical role of monocyte chemoattractant protein-1 receptor CCR2 on monocytes in hypertension-induced vascular inflammation and remodeling. *Circ Res*. 2004;94:1203–1210.
22. Sata M, Saiura A, Kunisato A, Tojo A, Okada S, Tokuhisa T, Hirai H, Makuuchi M, Hirata Y, Nagai R. Hematopoietic stem cells differentiate into vascular cells that participate in the pathogenesis of atherosclerosis. *Nat Med*. 2002;8:403–409.
23. Han KH, Han KO, Green SR, Quehenberger O. Expression of the monocyte chemoattractant protein-1 receptor CCR2 is increased in hypercholesterolemia. Differential effects of plasma lipoproteins on monocyte function. *J Lipid Res*. 1999;40:1053–1063.

Blockade of Vascular Endothelial Growth Factor Suppresses Experimental Restenosis After Intraluminal Injury by Inhibiting Recruitment of Monocyte Lineage Cells

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Background—Therapeutic angiogenesis by delivery of vascular endothelial growth factor (VEGF) has attracted attention. However, the role and function of VEGF in experimental restenosis (neointimal formation) after vascular intraluminal injury have not been addressed.

Methods and Results—We report herein that blockade of VEGF by soluble VEGF receptor 1 (*sFlt-1*) gene transfer attenuated neointimal formation after intraluminal injury in rabbits, rats, and mice. *sFlt-1* gene transfer markedly attenuated the early vascular inflammation and proliferation and later neointimal formation. *sFlt-1* gene transfer also inhibited increased expression of inflammatory factors such as monocyte chemoattractant protein-1 and VEGF. Intravascular VEGF gene transfer enhanced angiogenesis in the adventitia but did not reduce neointimal formation.

Conclusions—Increased expression and activity of VEGF are essential in the development of experimental restenosis after intraluminal injury by recruiting monocyte-lineage cells. (***Circulation***. **2004;110:2444-2452.**)

Key Words: restenosis ■ remodeling ■ inflammation ■ endothelium-derived factors ■ gene therapy

Vascular endothelial growth factor (VEGF) has attracted attention for endothelial regeneration and angiogenesis.[1–3] VEGF is one of the most potent vascular permeability factors known, is thought to function as an endogenous regulator of endothelial integrity after injury, and thus, protects the artery from disease progression.[4] Previous animals studies have reported that local delivery of VEGF after endothelial injury promotes endothelial regeneration, accelerates the recovery of endothelium-dependent relaxation, and reduces neointimal formation (see review[4]), suggesting the close correlation between accelerated endothelial integrity and reduced neointima after balloon injury. Increased expression of VEGF and its 2 receptors (VEGFR-1, Flt-1; VEFGR-2, Flk-1) in atherosclerotic and restenotic lesions has been reported.[5–7] However, there is still considerable debate over the vasculoprotective versus atherogenic effects of VEGF.[8] Emerging evidence suggests that (1) VEGF induces migration and activation of monocytes[9]; (2) VEGF induces adhesion molecules[10] and monocyte chemoattractant protein-1 (MCP-1)[11]; and (3) VEGF enhances neointimal formation and atherogenesis by stimulating intraplaque angiogenesis in hypercholesterolemic animals without balloon injury[12,13] or by increasing monocyte infiltration into atherosclerotic lesions.[14] Therefore, it remains unclear whether VEGF protects the artery from vascular disease or accelerates vascular disease.

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Clinical and experimental studies involving arterial gene transfer of VEGF showed that it failed to reduce restenosis after balloon angioplasty.[15–17] The role of VEGF in restenotic changes (neointimal formation and negative remodeling) after injury therefore remains a mystery. This is mainly because the inhibitor of VEGF has not been tested for experimental restenosis, although inhibitors of VEGF are currently being evaluated for tumor angiogenesis and other treatment-intractable inflammatory disorders.[3] It is practically impossible to investigate the role of VEGF in postnatal life in mice lacking VEGF or its receptors, because the absence of VEGF function leads to embryonic lethality owing to vascular defects.[4] A soluble form of the VEGF receptor-1 (sFlt-1) is expressed endogenously by vascular endothelial cells and can inhibit VEGF activity by directly sequestering VEGF and by functioning as a dominant-negative inhibitor against VEGF.[18] We and others have demonstrated that intramuscular transfection of the *sFlt-1* gene effectively and specifically

Received June 18, 2004; revision received August 14, 2004; accepted August 20, 2004.

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DOI: 10.1161/01.CIR.0000145123.85083.66

blocks VEGF and thus, "quenches" the activity of VEGF in remote organs in vivo.[19,20]

The aim of this study was to decisively determine a role for VEGF in restenotic changes after intraluminal injury. We report herein that blockade of VEGF by systemic *sFlt-1* gene transfer attenuates the development of neointimal formation after intraluminal injury by inhibiting inflammation, which suggests an essential role for VEGF in the pathogenesis of restenosis after injury. Our present data are clinically important because VEGF gene therapy for therapeutic angiogenesis and restenosis has been attempted in clinical studies.[16,17,21]

Methods

Expression Vector

The 3.3-kb mouse *sFLT-1* gene, originally obtained from the mouse lung DNA library,[22] was cloned into the *Bam*HI (5′) and *Not*1 (3′) sites of the eukaryotic expression vector plasmid cDNA3 (Invitrogen). Plasmid cDNA3 encoding the luciferase gene was used to detect gene transfection.

Rat and Rabbit Models of Balloon Injury

The study protocol was reviewed and approved by the Committee on Ethics on Animal Experiments, Kyushu University Faculty of Medicine, and the experiments were conducted according to the guidelines of American Physiological Society. A portion of this study was performed at the Kyushu University Station for Collaborative Research.

Twenty-week-old male, normal chow–fed Wistar-Kyoto rats were anesthetized, and their right common carotid arteries were injured by passage (3 times) of an infiltrated 2F Fogarty balloon catheter.[23] Male Japanese white rabbits weighing 3.0 to 3.5 kg were fed a high-cholesterol diet for 2 weeks. Their right common carotid arteries also were injured by passage (3 times) of an inflated 2F Fogarty catheter.[24] After injury, all rabbits were fed the same high-cholesterol diet. Three days before balloon injury, the animals were randomly divided into 2 groups: the empty-plasmid group was injected with the empty plasmid, and the *sFlt-1* group was injected with the *sFlt-1* gene into femoral muscle (150 μg/50 μL TE buffer [10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0] in rats, 1500 μg/0.5 mL TE buffer in rabbits). To enhance transgene expression, all plasmid-injected animals received electroporation at the injection site immediately after injection with an electric pulse generator (CUY21, BTX) as previously described.[19,23–25]

Morphometric and Immunohistochemical Analyses

All animals were euthanized by intravenous injection of a lethal dose of sodium pentobarbital. Tissue sections from rabbits and rats were prepared as described[23,24] and either (1) stained with Masson's trichrome or elastica van Gieson's stains or (2) subjected to immunostaining with antibodies against macrophages/monocytes (ED1, Serotec, for rats; RAM11, Dako, for rabbits), proliferating cells (proliferating cell nuclear antigen for rats from Dako, Ki-67 for rabbits from Dako), endothelial cells (CD31, Dako), VEGF (Santa Cruz), VEGFR-1 (Santa Cruz), VEGFR-2 (Santa Cruz), α-smooth muscle actin (Dako), MCP-1 (R&D Systems), interleukin-1β (IL-1β; R&D Systems), or nonimmune mouse IgG (Zymed). After avidin-biotin amplification, the slides were incubated with diaminobenzidine and counterstained with hematoxylin. Immunofluorescence double staining was performed to localize VEGF and its receptors by the use of fluorescence-conjugated antibodies in rats. Morphometric analysis was performed by microscopy with a computerized digital image-analysis system by a single observer who was blinded to the treatment protocol.[23,24]

Real-Time Quantitative Reverse Transcription–PCR

Real-time polymerase chain reaction (PCR) amplification was performed with rabbit cDNA by using the ABI PRISM 7000 sequence detection system (Applied Biosystems) as described previously.[23] The respective PCR primers and TaqMan probes were designed from GenBank databases aided by a software program (Applied Biosystems; online Table I). Results were analyzed by sequence detection software (Applied Biosystems), expressed in arbitrary units, and adjusted for glyceraldehyde 3-phosphate dehydrogenase mRNA levels.

Mouse Femoral Wire-Injury Model With Bone Marrow Reconstitution

Intraluminal injury of the femoral artery of wild-type mice whose bone marrow had been replaced with that of ROSA26 mice, which expresses β-galactosidase (LacZ) ubiquitously, was performed.[26] Four weeks after bone marrow transplantation, transluminal arterial injury was induced by inserting a straight spring wire (0.38 mm in diameter) into the femoral artery as described.[26] The femoral artery was excised and stained with X-gal solution for 7 hours and then fixed in 4% paraformaldehyde. LacZ-positive cells were counted and expressed as a proportion of the total number of cells. The paraffin-embedded sections were stained with elastica van Gieson's stain.

Peripheral blood was obtained from the retro-orbital venous plexus of the mice. Fluorescence-conjugated antibodies against CD31 (Pharmingen) and c-Kit (Pharmingen) were used as bone marrow–derived monocyte-lineage markers. A fluorescein isothiocyanate–conjugated antibody against Mac-1 (Pharmingen) was used as a circulating monocyte-lineage marker after gating for monocyte cell size with exclusion of granulocytes. Data were analyzed by flow cytometry and appropriate software (Becton Dickinson).

Blood Measurements

Plasma total cholesterol levels in rabbits were determined with commercially available kits (Wako Pure Chemicals). To measure sFlt-1 released by the transfected skeletal muscle, plasma concentrations of sFlt-1 were measured by use of an sFlt-1 ELISA kit (R&D Systems) in rabbits. Concentrations of VEGF in plasma and femoral arterial tissues were also measured in mice by use of an ELISA kit (R&D Systems).

Statistical Analysis

Data are expressed as mean±SE. Statistical analysis of differences was compared by ANOVA and Bonferroni's multiple-comparison tests. A level of $P<0.05$ was considered statistically significant.

Results

Increased Expression of VEGF and Its Receptors in Rabbits and Rats

Significant increases in VEGF mRNA levels were detected after balloon injury in rabbits, which peaked on day 7 and persisted until day 28 (Figure 1A). Immunohistochemical staining revealed that VEGF and VEGFR-1 increased in vascular smooth muscle cells in the media and regenerated endothelial layer during the early phase (day 7) and in cells in the neointima, media, and adventitia during the later phase (day 28) after balloon injury in rabbits (Figure 1B). VEGFR-2 did not increase on day 7 but did increase in the injured artery on day 28. *sFlt-1* gene transfer reduced the increased immunoreactivities of VEGF, VEGFR-1, and VEGFR-2 on day 28 (Figure 1B).

The localization of VEGF, VEGFR-1, and VEGFR-2 was studied in rats by immunohistochemistry. As observed in rabbits, immunoreactive VEGF and VEGFR-1 increased in the media on days 3 and 7 and in the neointima, media, and adventitia on day 28 (Figure 2A). The increase in immunoreactive VEGFR-2 was less prominent during the early phase but became apparent on day 28 (Figure 2A).

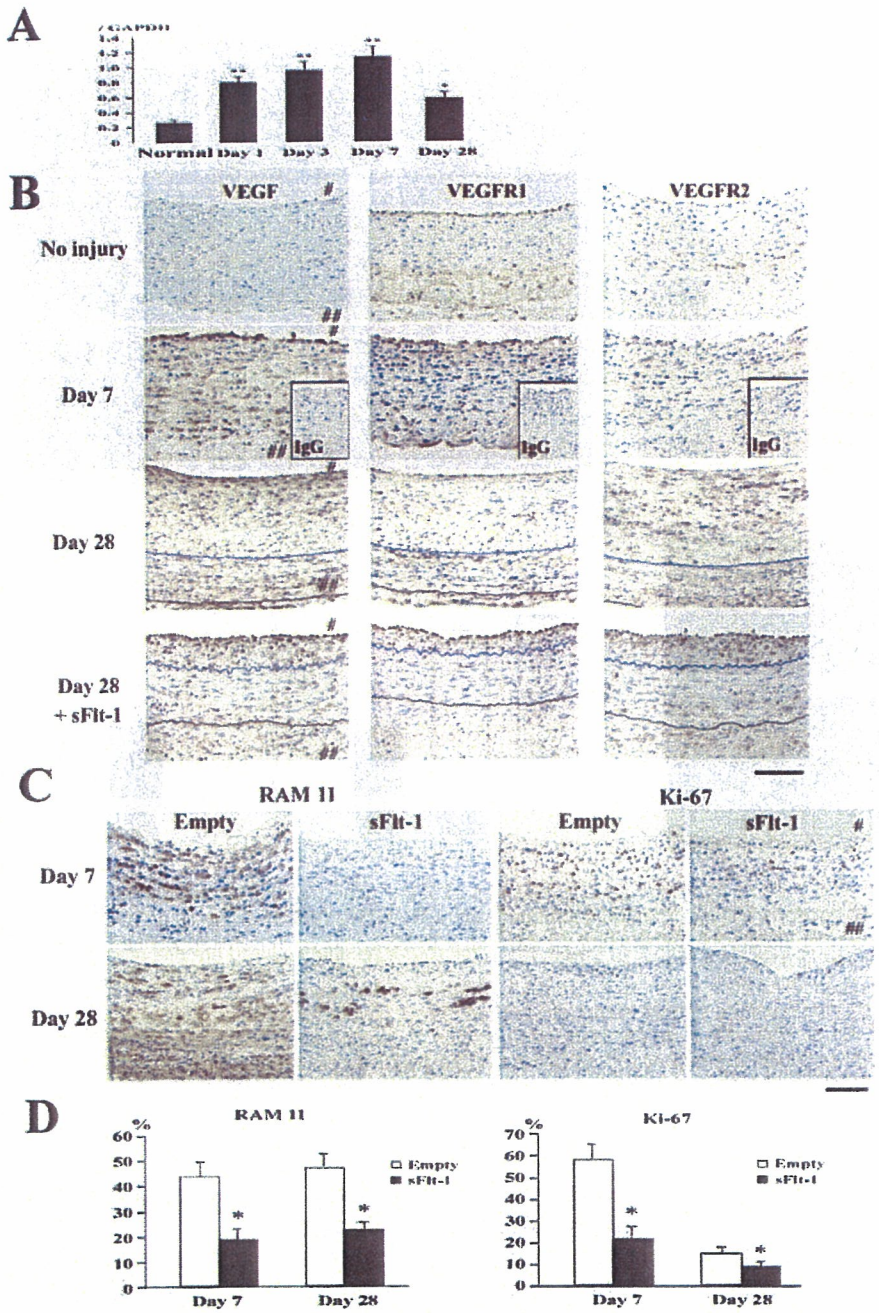

Figure 1. Expression of VEGF after balloon injury and inhibitory effects of *sFlt-1* gene transfer on inflammatory-proliferative changes in rabbits. A, Time course of VEGF mRNA levels in rabbits. mRNA levels were assessed by real-time PCR at indicated times. Expression of VEGF mRNA in each sample was normalized to glyceraldehyde 3-phosphate dehydrogenase mRNA expression in that sample. Each bar has n=6 to 8. *$P<0.05$, **$P<0.01$ vs control (uninjured) artery. B, Immunohistochemistry of rabbit carotid artery. Arterial cross sections were stained immunohistochemically with VEGF, VEGFR-1 (Flt-1), VEGFR-2 (Flk-1), or nonimmune IgG. # and ## indicate lumen and adventitia, respectively. Internal and external elastic layers are highlighted with blue and black lines, respectively, in photographs taken 28 days after injury. Bar indicates 100 μm. C, Carotid artery sections from empty-plasmid and *sFlt-1* groups 7 and 28 days after balloon injury were stained immunohistochemically with antibody against monocytes/macrophages (RAM11) or proliferating cells (Ki-67). # and ## indicate lumen and adventitia, respectively. Bar=100 μm. D, Effect of *sFlt-1* gene transfer on inflammation (RAM11-positive monocytes/macrophages) and proliferation (Ki-67–positive cells) 7 and 28 days after balloon injury (n=8 each). *$P<0.01$ vs control group.

Fluorescence double immunohistochemistry revealed that VEGF and VEGFR-1 were expressed predominantly in α-smooth muscle actin–positive cells in the media and neointima on day 28 (Figure 2B). During the early phase, ED1-positive monocytes recruited into the intima and adventitia expressed VEGFR-1 but not VEGFR-2 (Figure 2C). *sFlt-1* gene transfer reduced the increased immunoreactivities of VEGF, VEGFR-1, and VEGFR-2 on day 28 (data not shown).

Inhibitory Effects of *sFlt-1* Transfer on Inflammatory and Proliferative Changes in Rabbits

As we reported,[23,24] inflammatory and proliferative changes became evident by 3, 7, and 28 days after balloon injury in rabbits (Figure 1C and 1D). *sFlt-1* gene transfer reduced these inflammatory and proliferating changes.

Inhibitory Effects of *sFlt-1* Transfer on Neointimal Formation and/or Negative Remodeling in Rabbits and Rats

The carotid arteries in the control and empty-plasmid groups developed significant neointimal formation and negative remodeling (smaller lumen size, internal elastic lamina, and external elastic lamina) in rabbits by day 28 (Figure 3A and 3B). The arteries from the *sFlt-1* group showed less neointimal formation, negative remodeling, perivascular fibrosis, and adventitial vasa vasorum; an adventitial VEGFR-2–positive vasa vasorum; and a larger lumen area. There was no significant difference in plasma levels of total cholesterol between the 2 groups (online Table II), indicating that the observed effects of *sFlt-1* gene transfer were not caused by changes in serum cholesterol levels. In rats, neointimal

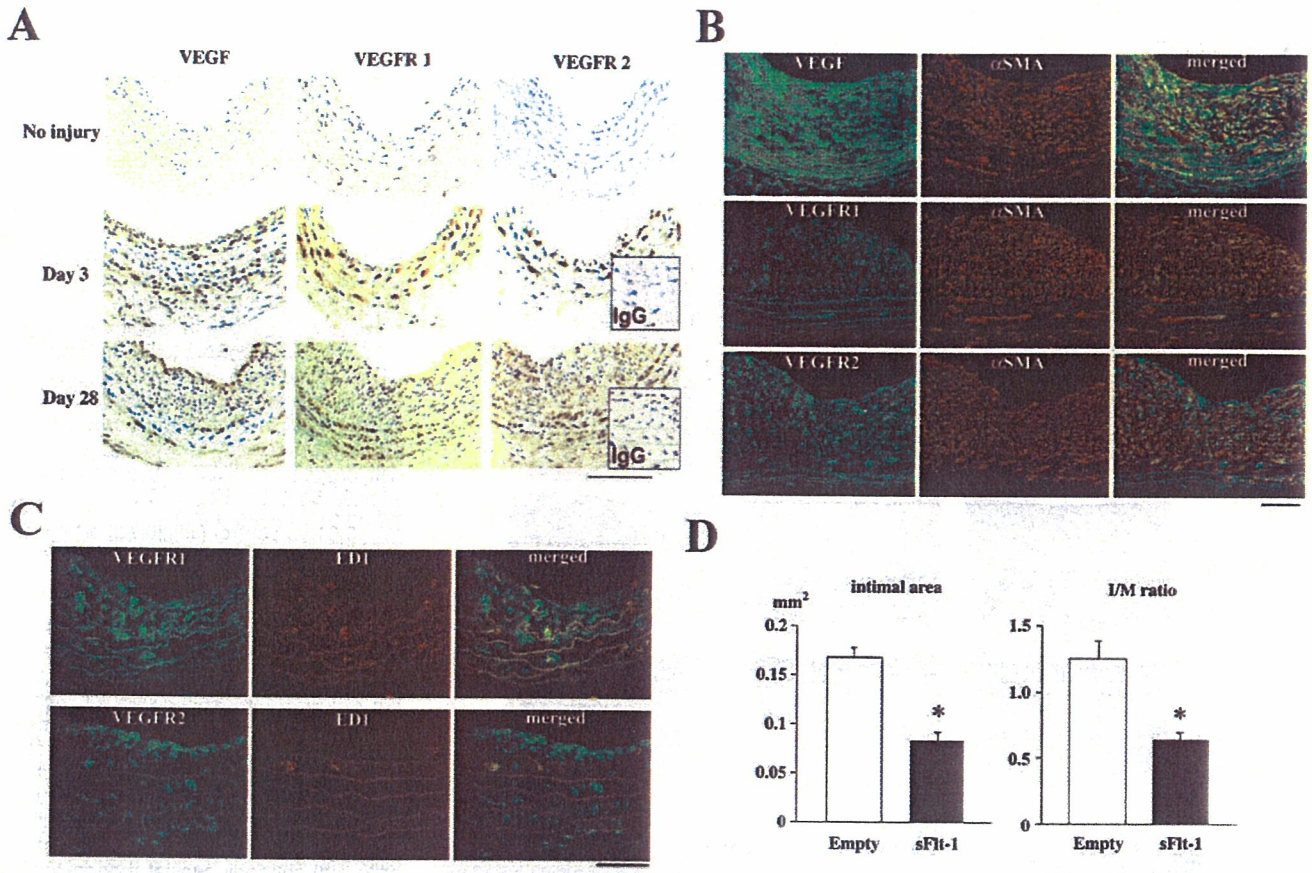

Figure 2. Expression of VEGF and its receptors after balloon injury and inhibitory effects of *sFlt-1* gene transfer on neointimal formation in rats. A, Immunohistochemistry of arterial cross-sections stained immunohistochemically with VEGF, VEGFR-1 (Flt-1), or VEGFR-2 (Flk-1). Bar indicates 100 μm. B, Fluorescence double immunohistochemistry 28 days after balloon injury. Photomicrographs show injured arteries stained with VEGF, VEGF-R1 (Flt-1), or VRGFR-2 (Flk-1) in green. Photomicrographs show injured arteries also stained with α-smooth muscle actin in red. Single-fluorescence–positive cells were stained in green or red, whereas double-positive cells were stained in yellow. Scale bar indicates 100 μm. C, Fluorescence double immunohistochemistry 7 days after balloon injury. Photomicrographs show injured arteries stained with VEGF-R1 or VEGF-R2 in green. Photomicrographs show injured arteries also stained with ED1 in red. Single-fluorescence–positive cells were stained in green or red, whereas double-positive cells were stained in yellow. Scale bar indicates 100 μm. D, Neointimal formation (neointimal area and intima-media ratio) on 28 day after balloon injury. *$P<0.01$ vs empty-plasmid group, n=8 or 9.

formation was also less in the *sFlt-1* group than in the empty-plasmid group on day 28 (Figure 2D).

To assess transfection efficacy of *sFlt-1*, plasma sFlt-1 concentration was measured in rabbits. The plasma sFlt-1 levels were 96±14, 377±25 ($P<0.01$ versus before), 413±20 ($P<0.01$), 284±15 ($P<0.05$), and 113±16 ($P>0.1$) pg/mL before and at 3, 7, 14, and 28 days after *sFlt-1* transfection, respectively, indicating that sFlt-1 was released from the transfected muscle to the circulation.

No Significant Effects of *sFlt-1* Gene Transfer on Endothelial Regeneration in Rabbits and Mice

In rabbits, there were no significant differences between the empty-plasmid and *sFlt-1*–transfected groups in the ratio of luminal surface area covered with endothelium (Figure 4A) and that of the CD31-positive endothelial layer 7 days after injury (Figure 4B). In mice, endothelial recovery was scarcely observed on day 7 (data not shown) but was noted equally in the 2 groups on day 14 (Figure 4C).

Inhibitory Effects of *sFlt-1* Transfer on Expression of Proinflammatory Factors

sFlt-1 transfection reduced the increased gene expression of MCP-1, platelet-derived growth factor, transforming growth factor-β, IL-1β, IL-6, tumor necrosis factor-α, matrix metalloproteinase-9, and VEGF (Figure 5A). *sFlt-1* transfer did not affect the increased gene expression of matrix metalloproteinase-1 and tissue factor. Immunohistochemical staining performed 7 days after balloon injury revealed increased immunoreactive MCP-1 and IL-1β in cells in the neointima and smooth muscle cells in the media, which were attenuated by *sFlt-1* gene transfer (Figure 5B).

Contribution of Bone Marrow Cells to the Effect of *sFlt-1* Gene Transfer in Mice

As reported,[26] a considerable proportion of neointimal and medial cells were LacZ-positive 28 days after injury in mice whose bone marrow expressed LacZ ubiquitously. Intimal area, intima-media ratio, and LacZ-positive cells were decreased in *sFlt-1*–transfected mice than in empty plasmid–

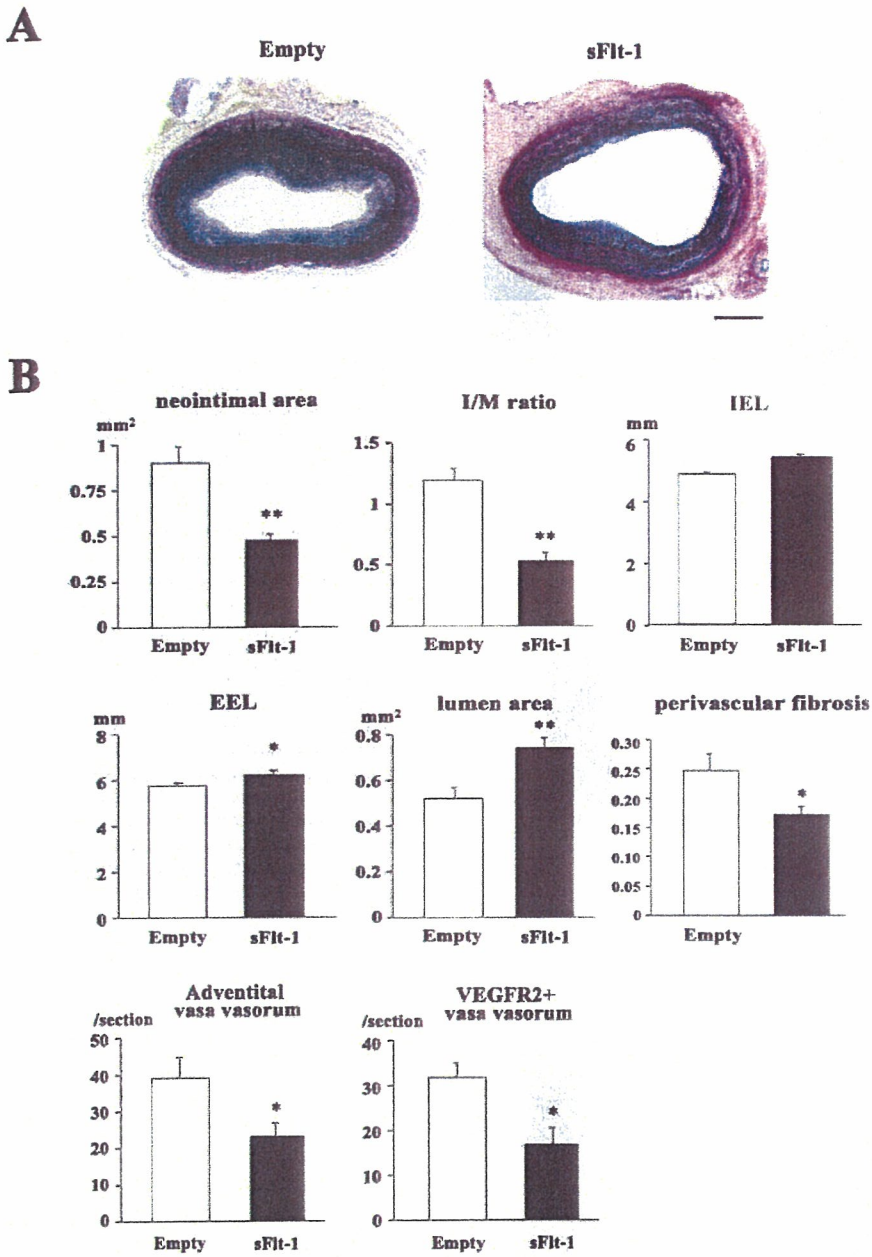

Figure 3. Inhibitory effect of *sFlt-1* gene transfer on restenotic changes (neointimal formation and negative remodeling) in rabbits. A, Carotid artery sections from empty-plasmid and *sFlt-1* groups 28 days after balloon injury stained with elastica van Gieson's stain. Bar=200 μm. B, Neointimal formation (neointimal area and intima-media ratio), negative remodeling (length of internal elastic lamina, length of external elastic lamina, and lumen area), perivascular fibrosis, adventitial vasa vasorum (number of CD31-positive vessels in adventitia), and VEGFR-2–positive vasa vasorum (number of VEGFR-2–positive vessels in adventitia) on day 28 after balloon injury are shown. *$P<0.05$, **$P<0.01$ vs empty-plasmid group, n=8 or 9.

transfected mice (Figure 6A and 6B). Wire injury also increased recruitment of bone marrow–derived monocytes (CD31-positive and c-Kit–positive) and circulating monocytes (Mac-1–positive) into peripheral blood (Figure 6C). *sFlt-1* gene transfer attenuated such changes in cell distribution, suggesting that wire injury increased such cell lineages in peripheral blood through VEGF. Plasma and femoral arterial concentrations of VEGF increased after wire injury, which was partly attenuated by *sFlt-1* transfection (online Tables III and IV).

Effects of $VEGF_{165}$ Gene Transfer on Neointimal Formation and Adventitial Angiogenesis in Rabbits

A recombinant adenoviral vector containing human $VEGF_{165}$ or the *LacZ* gene was produced. Gene transfer was performed by administering adenovirus solution (1 mL, 2×10^9 plaque-forming units) by a channel balloon catheter (Remedy, Boston Scientific Inc) immediately after balloon injury of rabbit carotid arteries (online Data Supplement and Figure). There were no significant differences between the empty-plasmid and *VEGF*-transfected groups in terms of neointimal formation, perivascular fibrosis, and negative remodeling (smaller lumen size, internal elastic lamina, and external elastic lamina) on day 28. In contrast, the number of adventitial vasa vasorum (the degrees of adventitial angiogenesis) was markedly increased in the *VEGF*-transfected group.

Discussion

VEGF has conventionally been thought to be an endothelial cell–specific growth factor and that it inhibits vascular pathological processes by accelerating endothelial proliferation and regeneration through endothelial VEGFR-2. If so, blockade of VEGF would suppress endothelial regeneration

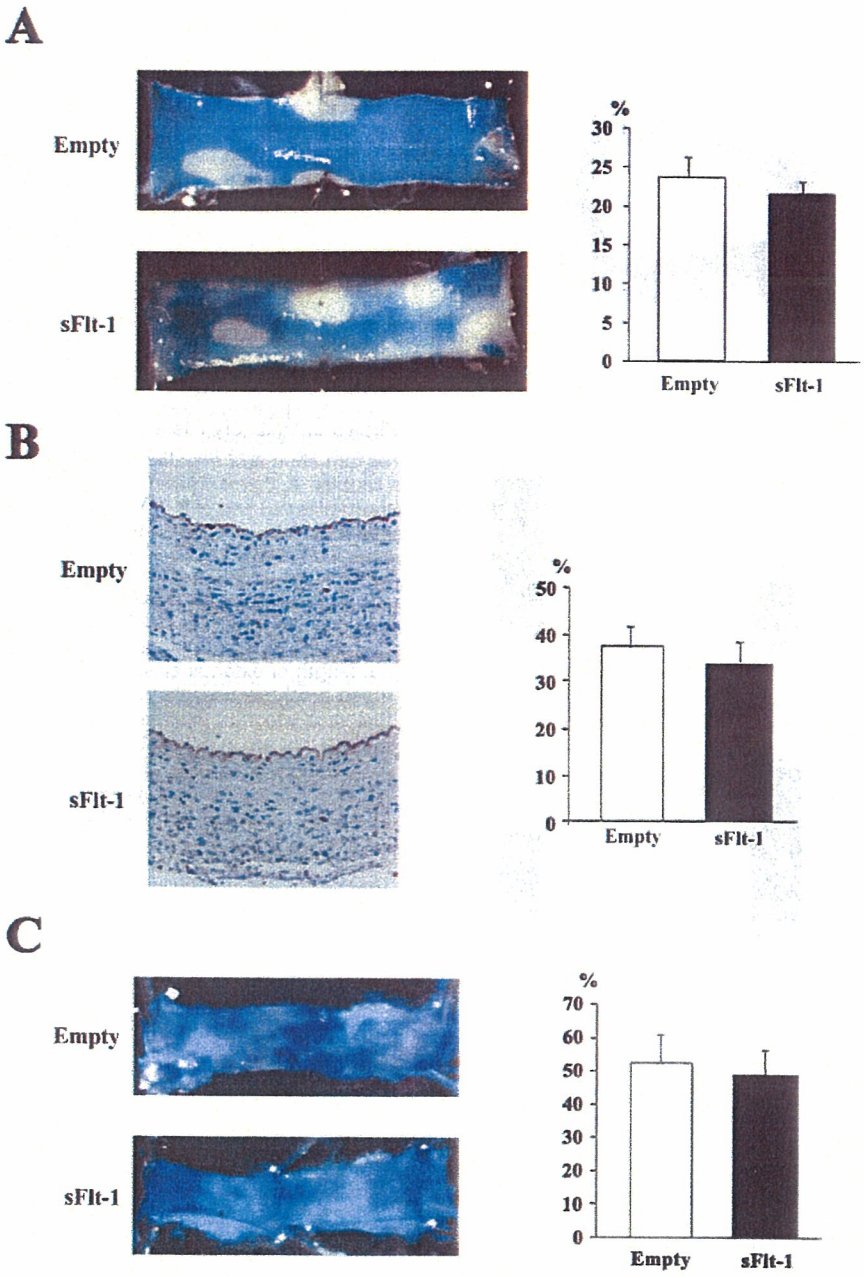

Figure 4. No significant effect of *sFlt-1* gene transfer on endothelial regeneration. A, Reendothelialization of rabbit carotid artery determined by Evans blue staining (deendothelialized areas are stained with blue) 7 days after balloon injury in rabbits. Endothelial regeneration of injured arteries was identified by intravenous injection of Evans blue dye 30 minutes before harvesting of vessels from rabbits and mice. Ratios of surface area covered by endothelium to total area in empty-plasmid and *sFlt-1* groups (n=7 each) are shown. B, Cross sections of rabbit femoral arteries stained with CD31 antibody 7 days after injury. Degrees of endothelial recovery (length of CD31-positive layer, length of internal elastic lamina in cross sections) in both groups are also shown (n=7 each). C, Reendothelialization of mouse femoral arteries 14 days after injury as determined by Evans blue staining (n=9 each).

and enhance neointimal formation after injury. In contrast to the conventional assumption, we here demonstrated that blockade of VEGF by *sFlt-1* gene transfer attenuated neointimal formation in rabbits, rats, and mice, indicating the essential role of VEGF in experimental restenosis.

As previously reported by others,[5–7,15] we demonstrated persistent increases in VEGF in arterial wall cells after injury. Emerging evidence suggests expression of functional VEGFR-1 and VEGFR-2 in cell types other than endothelial cells. We showed herein an increased expression of VEGFR-1 in lesional monocytes and medial smooth muscle cells during the early stage and in smooth muscle cells in the neointima and media during later stages. Increased VEGFR-2 expression was noted only at later stages. *sFlt-1* gene transfer attenuated the increased expression of inflammatory and growth factors such as VEGF, MCP-1, IL-1β, and so forth at early stages. Expression of VEGFR-1 in monocytes mediates chemotaxis,[9] and VEGFR-1 in smooth muscle cells mediates migration.[27] VEGFR-1 has been shown to act as an important mediator of VEGF-induced inflammation.[9,10,13] More recently, Yamada et al[28] showed that VEGF-mediated angiogenesis and inflammation are mediated by MCP-1. We also demonstrated the central role of MCP-1 in the mechanism of neointimal formation after vascular injury.[23–25,29] It is likely, therefore, that VEGF might cause vascular inflammation and migration of vascular smooth muscle cells and thus, cause neointimal formation after injury. Further studies are needed to elucidate the relative roles of VEGFR-1 and VEGFR-2 in the mechanisms of neointimal formation.

This study also demonstrated in rabbits the role of VEGF in the development of negative remodeling, another major cause of human restenosis after balloon angioplasty.[30] Fibro-

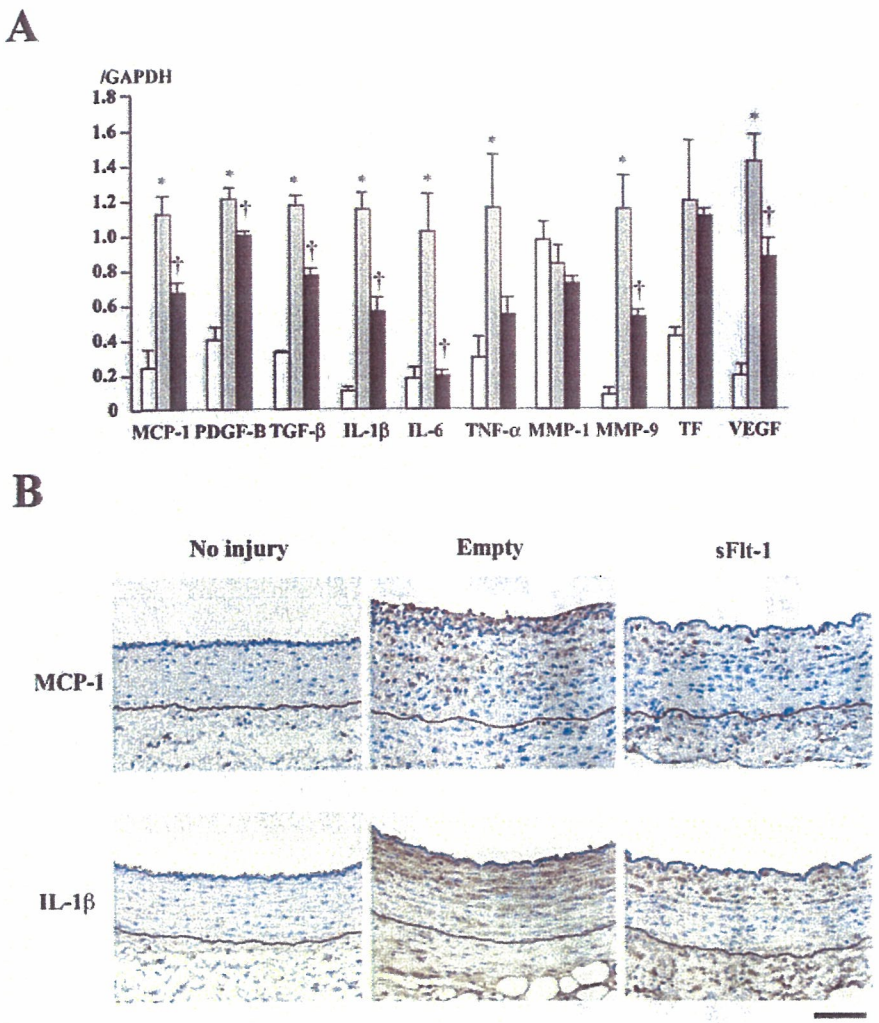

Figure 5. Inhibitory effect of *sFlt-1* gene transfer on expression of various inflammatory factors in rabbits. A, Effect of *sFlt-1* gene transfer on mRNA levels of various proinflammatory factors 1 day after injury. Quantitative real-time PCR was performed. **P*<0.01 vs uninjured control (uninjured) artery; †*P*<0.05, ††*P*<0.01 vs empty-plasmid group. B, Carotid artery sections from control uninjured animals and those from empty-plasmid and *sFlt-1* groups 7 days after balloon injury stained immunohistochemically with MCP-1 and IL-1β. Internal and external elastic layers are highlighted with blue and black lines, respectively. Bar=100 μm. Immunohistochemical experiments were repeated 5 times, all with representative results.

sis and vasa vasorum in the adventitia have been implicated to be the central pathological processes leading to constrictive negative remodeling after balloon injury. Therefore, our present data suggest that *sFlt-1* gene transfer inhibited the development of negative constrictive remodeling by limiting adventitial fibrosis and angiogenesis.

VEGFR-1 has been shown to be an important mediator of stem cell recruitment and differentiation.[13] Sata and colleagues[26] have shown that a considerable proportion of neointimal and medial cells were bone marrow–derived progenitor cells. However, the role of VEGF in the recruitment and differentiation of progenitor cells into neointimal cells after vascular injury has not been addressed. We showed here that *sFlt-1* gene transfer inhibited recruitment of bone marrow–lineage cells into the peripheral circulation and injured arterial wall and thus, reduced neointimal formation after injury. These data suggest that VEGF might contribute to neointimal formation by recruiting bone marrow–derived and circulating monocytes.

Surprisingly, *sFlt-1* gene transfer did not affect endothelial regeneration after endothelial injury, suggesting a minor role of endogenous VEGF in endothelial regeneration after endothelial injury. It remains to be determined whether inhibition of VEGFR-2–mediated activity of endothelial regeneration by *sFlt-1* gene transfer might have been overshadowed by other stimuli (eg, basic fibroblast growth factor, angiopoietins, etc). On the contrary, adenovirus-mediated gene transfer of *VEGF* enhanced adventitial angiogenesis but did not reduce neointimal formation after balloon injury in rabbits. The latter observation is consistent with previous reports demonstrating that exogenous VEGF does not reduce neointimal formation in animals and humans.[15–17] Taken together, the role or mechanisms of action of VEGF may differ between exogenous and endogenous VEGF and between angiogenesis and neointimal formation.

This study may have significant clinical implications. First, *sFlt-1* gene transfer might be an attractive anti-VEGF therapy for inflammatory vascular disease and other inflammatory disorders. However, local delivery of *sFlt-1* must be preferable for clinical use to avoid potential side effects by systemic delivery. Second, our finding indicates that more research is required, especially on the safety of VEGF, before translational clinical research proceeds. Deleterious effects associated with overexpression of VEGF have recently been reported: (1) injection of VEGF-expressing skeletal muscle myoblasts into the murine heart caused the formation of hemangiomas and lethagenic heart failure[31] and (2) *VEGF* gene transfer into rabbit carotid arteries induced neointimal

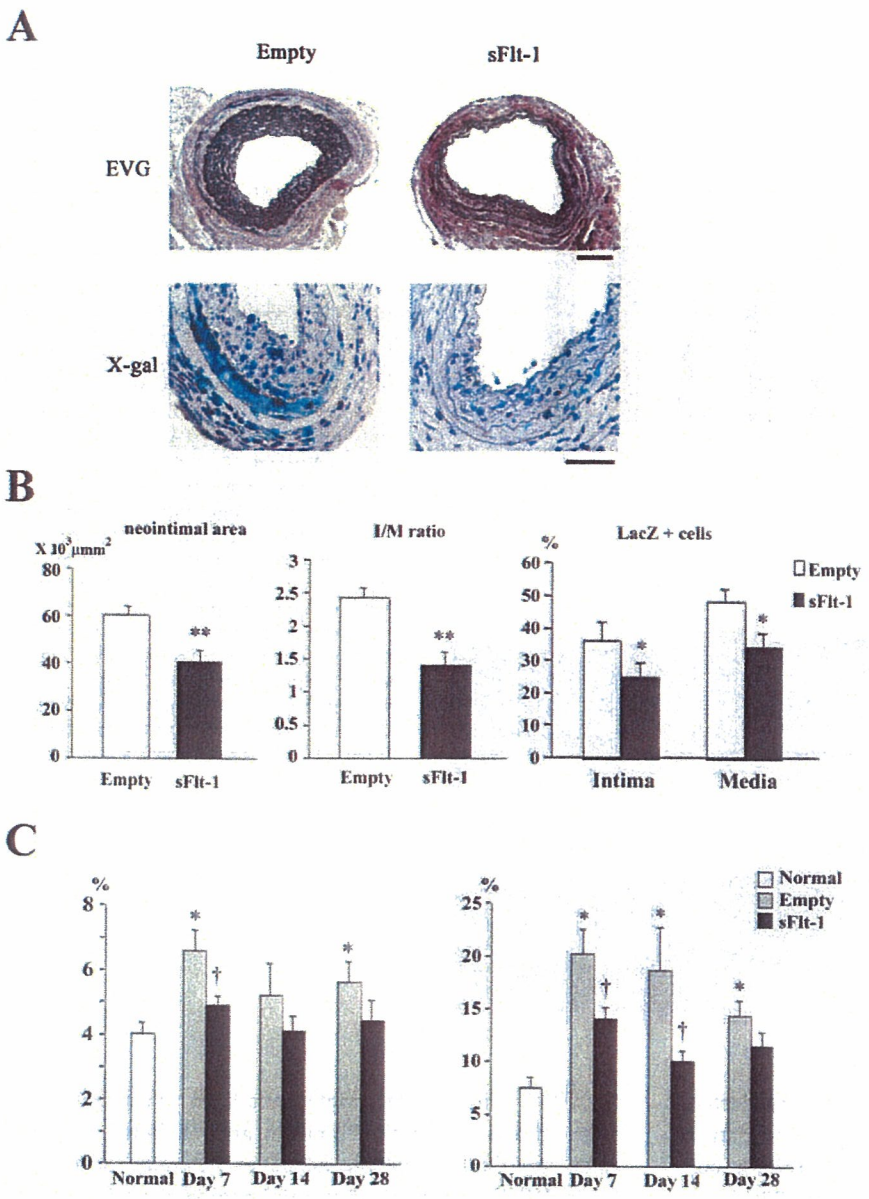

Figure 6. Contribution of bone marrow–derived cells to effect of *sFlt-1* gene transfer in mice. A, Arterial sections from empty-plasmid and *sFlt-1* groups 28 days after wire injury stained with X-gal or elastica van Gieson's (EVG) stains. Bar=100 μm. B, Inhibitory effects of *sFlt-1* gene transfer on neointimal formation (neointimal area and intima-media ratio) and percentage of LacZ-positive cells (100×LacZ-positive nuclei/total nuclei) in neointima and media. **P*<0.05, ***P*<0.01 vs phosphate-buffered saline, n=8 each. C, Summary of fluorescence-activated cell sorting analysis of recruitment of bone marrow–derived monocytes (left) and circulating monocytes (right) into peripheral circulation in normal mice and mice transfected with empty plasmid or *sFlt-1* after wire injury, n=7 or 8. **P*<0.05 vs normal mice (no injury); †*P*<0.05 vs empty-plasmid group.

formation with angiomatoid proliferation of endothelial cells.[12] These studies highlight the potential side effects or toxicity that would against the clinical use of VEGF for therapeutic angiogenesis and restenosis.

In conclusion, this study has provided direct in vivo evidence suggesting that increased expression and activity of VEGF are essential for the development of experimental restenosis after intraluminal injury by recruiting monocyte-lineage cells. Although there is no clinical evidence suggesting enhancement of atherosclerosis or neointimal formation by VEGF therapy,[16,17] our present data raise the question of whether VEGF therapy is useful without serious risks in patients with advanced atherosclerosis.

Acknowledgments

This study was supported by grants-in-aid for scientific research (14657172, 14207036) from the Ministry of Education, Science, and Culture, Tokyo, Japan; by health science research grants (Comprehensive Research on Aging and Health and Research on Translational Research) from the Ministry of Health Labor and Welfare, Tokyo, Japan; and by the Program for Promotion of Fundamental Studies in Health Sciences of the Organization for Pharmaceutical Safety and Research, Tokyo, Japan.

References

1. Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med.* 1995;1:27–31.
2. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature.* 2000;407:249–257.
3. Ferrara N, Alitalo K. Clinical applications of angiogenic growth factors and their inhibitors. *Nat Med.* 1999;5:1359–1364.
4. Baumgartner I, Isner JM. Somatic gene therapy in the cardiovascular system. *Annu Rev Physiol.* 2001;63:427–450.
5. Inoue M, Itoh H, Ueda M, et al. Vascular endothelial growth factor (VEGF) expression in human coronary atherosclerotic lesions: possible pathophysiological significance of VEGF in progression of atherosclerosis. *Circulation.* 1998;98:2108–2116.
6. Chen YX, Nakashima Y, Tanaka K, et al. Immunohistochemical expression of vascular endothelial growth factor/vascular permeability factor in atherosclerotic intimas of human coronary arteries. *Arterioscler Thromb Vasc Biol.* 1999;19:131–139.
7. Shibata M, Suzuki H, Nakatani M, et al. The involvement of vascular endothelial growth factor and flt-1 in the process of neointimal prolif-

eration in pig coronary arteries following stent implantation. *Histochem Cell Biol.* 2001;116:471–481.
8. Isner JM. Still more debate over VEGF. *Nat Med.* 2001;7:639–640.
9. Barleon B, Sozzani S, Zhou D, et al. Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1. *Blood.* 1996;87:3336–3343.
10. Kim I, Moon SO, Hoon Kim S, et al. Vascular endothelial growth factor expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin through nuclear factor-κB activation in endothelial cells. *J Biol Chem.* 2001;276: 7614–7620.
11. Marumo T, Schini-Kerth VB, Busse R. Vascular endothelial growth factor activates nuclear factor-κB and induces monocyte chemoattractant protein-1 in bovine retinal endothelial cells. *Diabetes.* 1999;48: 1131–1137.
12. Yonemitsu Y, Kaneda Y, Morishita R, et al. Characterization of in vivo gene transfer into the arterial wall mediated by the Sendai virus (hemagglutinating virus of Japan) liposomes: an effective tool for the in vivo study of arterial diseases. *Lab Invest.* 1996;75:313–323.
13. Luttun A, Tjwa M, Moons L, et al. Revascularization of ischemic tissues by PlGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1. *Nat Med.* 2002;8:831–40.
14. Celletti FL, Waugh JM, Amabile PG, et al. Vascular endothelial growth factor enhances atherosclerotic plaque progression. *Nat Med.* 2001;7: 425–429.
15. Hiltunen MO, Laitinen M, Turunen MP, et al. Intravascular adenovirus-mediated VEGF-C gene transfer reduces neointima formation in balloon-denuded rabbit aorta. *Circulation.* 2000;102:2262–2268.
16. Hedman M, Hartikainen J, Syvanne M, et al. Safety and feasibility of catheter-based local intracoronary vascular endothelial growth factor gene transfer in the prevention of postangioplasty and in-stent restenosis and in the treatment of chronic myocardial ischemia: phase II results of the Kuopio Angiogenesis Trial (KAT). *Circulation.* 2003;107: 2677–2683.
17. Henry TD, Annex BH, McKendall GR, et al. The VIVA trial: Vascular endothelial growth factor in Ischemia for Vascular Angiogenesis. *Circulation.* 2003;107:1359–1365.
18. Kendall RL, Wang G, Thomas KA. Identification of a natural soluble form of the vascular endothelial growth factor receptor, FLT-1, and its heterodimerization with KDR. *Biochem Biophys Res Commun.* 1996;226: 324–328.
19. Zhao Q, Egashira K, Inoue S, et al. Vascular endothelial growth factor is necessary in the development of arteriosclerosis by recruiting/activating monocytes in a rat model of long-term inhibition of nitric oxide synthesis. *Circulation.* 2002;105:1110–1115.
20. Goldman CK, Kendall RL, Cabrera G, et al. Paracrine expression of a native soluble vascular endothelial growth factor receptor inhibits tumor growth, metastasis, and mortality rate. *Proc Natl Acad Sci U S A.* 1998; 95:8795–8800.
21. Losordo DW, Vale PR, Hendel RC, et al. Phase 1/2 placebo-controlled, double-blind, dose-escalating trial of myocardial vascular endothelial growth factor 2 gene transfer by catheter delivery in patients with chronic myocardial ischemia. *Circulation.* 2002;105:2012–2018.
22. Kondo K, Hiratsuka S, Subbalakshmi E, et al. Genomic organization of the flt-1 gene encoding for vascular endothelial growth factor (VEGF) receptor-1 suggests an intimate evolutionary relationship between the 7-Ig and the 5-Ig tyrosine kinase receptors. *Gene.* 1998;208:297–305.
23. Usui M, Egashira K, Ohtani K, et al. Anti-monocyte chemoattractant protein-1 gene therapy inhibits restenotic changes (neointimal hyperplasia) after balloon injury in rats and monkeys. *FASEB J.* 2002;16: 1838–1840.
24. Mori E, Komori K, Yamaoka T, et al. Essential role of monocyte chemoattractant protein-1 in development of restenotic changes (neointimal hyperplasia and constrictive remodeling) after balloon angioplasty in hypercholesterolemic rabbits. *Circulation.* 2002;105:2905–2910.
25. Egashira K, Zhao Q, Kataoka C, et al. Importance of monocyte chemoattractant protein-1 pathway in neointimal hyperplasia after periarterial injury in mice and monkeys. *Circ Res.* 2002;90:1167–1172.
26. Sata M, Saiura A, Kunisato A, et al. Hematopoietic stem cells differentiate into vascular cells that participate in the pathogenesis of atherosclerosis. *Nat Med.* 2002;8:403–409.
27. Wang H, Keiser JA. Vascular endothelial growth factor upregulates the expression of matrix metalloproteinases in vascular smooth muscle cells: role of flt-1. *Circ Res.* 1998;83:832–840.
28. Yamada M, Kim S, Egashira K, et al. Molecular mechanism and role of endothelial monocyte chemoattractant protein-1 induction by vascular endothelial growth factor. *Arterioscler Thromb Vasc Biol.* 2003;23: 1996–2001.
29. Egashira K. Molecular mechanisms mediating inflammation in vascular disease: special reference to monocyte chemoattractant protein-1. *Hypertension.* 2003;41:834–841.
30. Kakuta T, Currier JW, Haudenschild CC, et al. Differences in compensatory vessel enlargement, not intimal formation, account for restenosis after angioplasty in the hypercholesterolemic rabbit model. *Circulation.* 1994;89:2809–2815.
31. Lee RJ, Springer ML, Blanco-Bose WE, et al. VEGF gene delivery to myocardium: deleterious effects of unregulated expression. *Circulation.* 2000;102:898–901.

Essential Role of Vascular Endothelial Growth Factor and Flt-1 Signals in Neointimal Formation After Periadventitial Injury

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Objective—Vascular endothelial growth factor (VEGF) is upregulated after arterial injury. Its role in the pathogenesis of neointimal formation after periadventitial injury, however, has not been addressed.

Methods and Results—Expression of VEGF and its receptors but not that of placental growth factor markedly increased with the development of neointimal formation in hypercholesterolemic mice after cuff-induced periarterial injury. Transfection with the murine soluble Flt-1 (sFlt-1) gene to block VEGF in vivo in mice inhibited early inflammation and later neointimal formation. The sFlt-1 gene transfer did not affect plasma lipid levels but attenuated increased expression of VEGF, Flt-1, Flk-1, monocyte chemoattractant protein-1, and other inflammation-promoting factors. Mice with Flt-1 kinase deficiency also displayed reduced neointimal formation.

Conclusions—Inflammatory changes mediated by VEGF and Flt-1 signals play an important role in the pathogenesis of neointimal formation after cuff-induced periadventitial injury. VEGF might promote neointimal formation by acting as a proinflammatory cytokine. (*Arterioscler Thromb Vasc Biol.* 2004;24:2284-2289.)

Key Words: remodeling ■ growth substances ■ inflammation ■ arteriosclerosis ■ gene therapy

Neointimal formation is a major cause of restenosis after coronary intervention.[1,2] Vascular endothelial growth factor (VEGF) and its receptors (VRGFR-1: Flt-1, VEGFR-2: Flk-1) are upregulated in vascular inflammatory and proliferative disorders such as atherosclerosis and restenosis.[3–6] VEGF is thought to protect the artery from such disorders by inducing endothelial regeneration and improving endothelial function.[7] VEGF gene transfer or administration of its protein induces endothelial regeneration and attenuates neointimal formation after endothelial injury.[7–9] VEGF is reported to inhibit leukocyte infiltration through hemeoxygenase-1.[10] There is still considerable debate, however, over the role of VEGF in the development of neointimal formation after injury.[11,12] Emerging evidence suggests that VEGF causes or promotes the development of atherosclerosis or neointimal formation after injury. VEGF induces migration and activation of monocytes,[13] adhesion molecules,[14] or monocyte chemoattractant protein-1 (MCP-1)[15] through its receptor Flt-1. Moreover, administration of VEGF protein to hypercholesterolemic animals enhances atherogenesis by inducing monocyte infiltration and activation.[16] In addition, VEGF might promote migration of vascular smooth muscle cells though Flt-1.[17,18] Angiogenesis inhibitors are shown to reduce intimal neovascularization and plaque growth in hyperlipidemic mice.[19]

One major reason for the inconsistent reports regarding the role of VEGF might be because there are no selective VEGF inhibitors tested. The only known endogenous VEGF inhibitor is a soluble form of the VEGF receptor-1, Flt-1 (sFlt-1).[20] This isoform is mainly expressed by vascular endothelial cells and can inhibit VEGF activity by directly sequestering VEGF and by functioning as a dominant-negative inhibitor.[20] We and others previously demonstrated that intramuscular transfection of the sFlt-1 gene blocks VEGF signaling and thus quenches VEGF activity in vivo.[21,22] Therefore, sFlt-1 gene transfer can be used as an inhibitor against VEGF and its receptors (Flt-1, Flk-1). In addition, Flt-1 tyrosine kinase–deficient mice can be used to determine the role of Flt-1 signals.[23]

In this study, we investigated the role of VEGF and Flt-1 signals in the pathogenesis of neointimal formation after cuff-induced periadventitial injury in hypercholesterolemic mice. Several animal models for evaluation of neointimal formation after injury have been reported, including balloon injury, wire injury, chemical injury, and cuff injury, among others. The ideal animal model for human neointimal forma-

Original received October 21, 2003; final version accepted September 22, 2004.

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Consulting Editor for this article was Peter Libby, MD, Brigham and Women's Hospital, Boston, Mass.

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Arterioscler Thromb Vasc Biol. is available at http://www.atvbaha.org

DOI: 10.1161/01.ATV.0000147161.42956.80

tion is uncertain. The cuff model was chosen because cuff placement in the presence of hypercholesterolemia offers the advantage of inducing reproducible site-controlled neointimal formation and stenosis.[24,25] In addition, the cuff-induced injury triggers vascular inflammation and induces neointimal lesions that are partly similar to the restenotic and atherosclerotic lesions observed in humans.[24,25] Our present data provide direct evidence suggesting that inflammatory changes mediated by VEGF and Flt-1 signals play an important role in the pathogenesis of neointimal formation after cuff-induced periadventitial injury.

Methods

Expression Vector

The 3.3-kb mouse sFlt-1 gene was obtained from a mouse lung cDNA library[26] and cloned into the BamHI(5′) and NotI(3′) sites of the eukaryotic expression vector plasmid cDNA3 (Invitrogen).

Experimental Animals

The study protocol was reviewed and approved by the Committee on Ethics for Animal Experiments, Kyushu University Faculty of Medicine, and the experiments were conducted according to the Guidelines of the American Physiological Society. A part of this study was performed at the Kyushu University Station for Collaborative Research.

Apolipoprotein E–deficient (apoE-KO) and wild-type mice (8 to 10 weeks old, n=5 to 9 each group) with a genetic background of C57BL/6J were purchased from The Jackson Laboratory (Bar Harbor, Me) and fed with commercial standard chow. Placement of cuff and gene transfer were performed as previously described.[21,27] A nonconstrictive polyethylene cuff (1.5 mm long; PE20, 0.38-mm inner diameter, 1.09-mm outer diameter) was placed loosely around the left femoral artery. Either empty plasmid or sFlt-1 plasmid (300 μg/100 μL PBS) was injected into the right femoral muscle using a 27-gauge needle immediately and 10 days after cuff placement. To enhance transgene expression, these animals received electroporation at the injected site immediately after injection.[21,27–29] It has been shown that electroporation-mediated gene transfer is useful to introduce genes into muscle tissues in vivo with no serious tissue injury.[30] To determine the role of flt-1 signals, Flt-1 tyrosine kinase–deficient mice with a genetic background of C57BL/6J were used.[23]

In Vivo Matrigel Plug Assay

An in vivo matrigel plug assay was used to determine the effect of sFlt-1 gene transfer on VEGF activity.[21,27] Matrigel matrix alone (300 μL) or mixed with recombinant VEGF protein (100 ng/mL) was injected subcutaneously into the flanks of C57BL/6J mice. The matrigels were then removed 7 or 14 days after injection, and angiogenesis and inflammation were examined by histopathologic analysis.

Histopathology, Immunohistochemistry, and Morphometry

Mice were anesthetized with pentobarbital, and the femoral artery was harvested, fixed overnight in 3.7% formaldehyde in PBS, and paraffin-embedded.[27] Serial cross sections (5 μm thick) throughout the entire length of the cuffed femoral artery were used for histological analysis. Cryosections were made from 2 mice in each condition. All sections were routinely stained with hematoxylin-eosin or van Gieson. Mac-3 (PharMingen) staining was used to detect monocytes/macrophages, and CD3 (Santa Cruz Biotechnology) was used for T cells. Proliferating cell nuclear antigen (PCNA; Santa Cruz Biotechnology) was used to detect vascular proliferation. An antibody against von Willebrand factor (vWF; Sigma Chemical Co) was used to mark endothelial cells. Antibodies against VEGF (Santa Cruz Biotechnology) and placental growth factor (PlGF; R&D Systems Inc) were also used. Indirect immunofluorescence double-staining with matched primary and fluorescein-conjugated secondary antibodies was used to stain for colocalization with VEGF receptors in smooth muscle cells or monocytes as follows: Rabbit anti-mouse Flt-1 (Santa Cruz Biotechnology), rabbit anti-mouse Flk-1 (Santa Cruz Biotechnology), rat anti-mouse Mac-3, anti–α-smooth muscle actin (α-SMA; Boehringer Mannheim Corp), anti-rabbit IgG conjugated with fluorescein isothiocyanate or rhodamine, and anti-rat IgG conjugated with fluorescein isothiocyanate or rhodamine (Santa Cruz Biotechnology).

Ten equally-spaced cross sections were examined in all mice to quantify intimal lesions. Using image analysis software, the total cross-sectional medial area was measured between the external and internal elastic lamina; the total cross-sectional intimal area was measured between the endothelial cell monolayer and the internal elastic lamina.

Plasma Measurements

Plasma total cholesterol and triacylglycerol levels were determined with commercially available kits (Wako Pure Chemicals). Plasma concentrations of sFlt-1, VEGF, and PlGF were measured by the use of ELISA kit (R&D Systems Inc).

RT-PCR and RNAse Protection Assay

RNA was prepared from the pooled samples (5 to 7 arteries for 1 sample).[21] First-strand DNA was synthesized using reverse transcriptase with random hexamers from 1 μg total RNA in a 20-μL reaction volume according to the manufacturer's protocol (GeneAmp RNA polymerase chain reaction Kit; Perkin–Elmer). Primers used for amplification of VEGF were 5′-GGA TCC ATG AAC TTT CTG CT-3′ and 5′-GAA TTC ACC GCC TCG GCT TGT C-3′ with expected sizes of 654, 582, and 450 bp for the 3 VEGF isoforms (VEGF 188, 164, and 120, respectively). Primers for PlGF were 5′-CCC ACA CCC AGC TCA CGT ATT TA-3′ and 5′-TCC CCT CTA CAT GCC TTC AAT GC-3′. Primers for Flk-1 were 5′-ACT GCA GTG ATT GCC ATG TTC T-3′ and 5′-GCT CAT CCA AGG GCA ATT CAT-3′. Primers for the internal control, β-actin, were 5′-ATG GAT GAC GAT ATC GCT-3′ and 5′-ATG AGG TAG TCT GCT AGG T-3′ with an expected product of 550 bp.

RNAse protection assays were performed using 5 μg total RNA with 2 custom template sets according to the manufacturer's protocol (PharMingen).[27]

Statistical Analysis

Data are expressed as the mean±SE. Statistical analysis of differences was compared by ANOVA. Post hoc analyses were performed using Bonferroni correction for multiple comparison tests. $P<0.05$ was considered to be statistically significant.

Results

In Vivo Matrigel Plug Assay in ApoE-KO Mice

Seven days after injection of matrigel, there were significant angiogenic (number of CD31-positive cells per mm^2) and inflammatory (number of Mac3-positive cells per mm^2) reactions in the matrigel plugs containing recombinant VEGF protein compared with matrigel alone. Soluble Flt-1 gene transfer but not injection of an empty plasmid suppressed both the angiogenic and inflammatory reactions to VEGF to a level similar to that of matrigel plugs without VEGF (Figure I, available online at http://atvb.ahajournals.org). This suppression of angiogenic and inflammatory reactions to VEGF was noted on day 14 but not on day 21 of sFlt-1 gene transfer (data not shown).

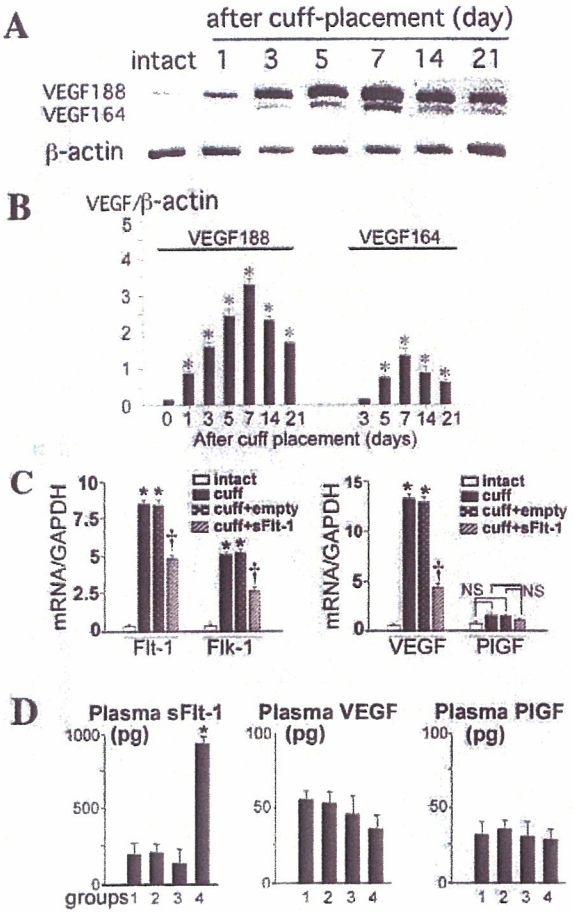

Figure 1. Gene expression VEGF, Flt-1, Flk-1, and PlGF in cuffed femoral artery. A, Time course of VEGF mRNA levels (RT-RCR) and expression of arterial VEGF and β-actin mRNA after cuff placement. mRNA levels were assessed at the indicated times. This is a representative assay from 5 separate experiments. B, Densitometric analysis of data in A. Expression of VEGF mRNA in each sample was normalized by β-actin mRNA expression in the same sample. N=5 for each bar. *$P<0.01$ vs control intact artery. C, Gene expressions of Flt-1, Flk-1, PlGF, and VEGF (RT-PCR) in femoral arteries before or 7 days after cuff placement/sFlt-1 gene transfer. *$P<0.01$ vs intact control. D, Plasma levels of sFlt-1, PlGF, and VEGF 7 days after cuff placement in 4 animal groups: (1) untreated control, (2) mice with cuff alone, (3) cuff+empty plasmid, and (4) cuff+sFlt-1 plasmid. N=6 for each. *$P<0.01$ vs control group.

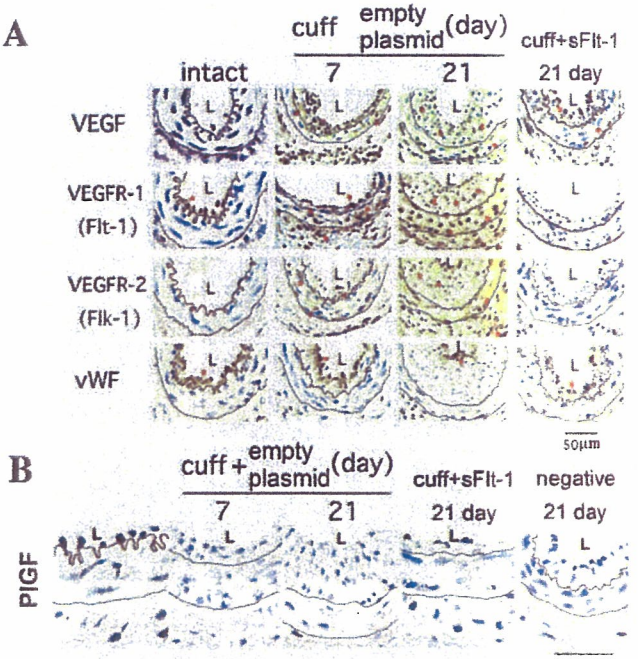

Figure 2. Immunostaining of VEGF, Flt-1, Flk-1, and PlGF in cuffed femoral artery. A, Cross sections of intact or cuffed femoral arteries were stained immunohistochemically against VEGF, VEGF receptor 1 (Flt-1), VEGF receptor 2 (Flk-1), or vWF 7 and 21 days after cuff placement in empty plasmid group. Immunohistochemical sections of cuffed arteries on day 21 in sFlt-1 group are also shown. Black lines indicate internal and external elastin laminas. L indicates lumen. Scale bar=50 μm. B, Immunohistochemical staining of PlGF in femoral arteries before or after cuff placement/sFlt-1 gene transfer. Black lines indicate internal or external elastin laminas. L indicates lumen. Scale bar=50 μm.

Gene Expression and Immunoreactivity in ApoE-KO Mice

The mRNA levels of 2 VEGF isoforms (188 and 164) markedly increased after cuff placement, whereas they were undetectably low in control intact artery (Figure 1A and 1B). Peak expression was observed on day 7. VEGF 121 mRNA was undetectable before and after cuff placement. Gene expression of Flt-1, Flk-1, VEGF, and PlGF was also increased on day 7 (Figure 1C). Plasma concentrations of sFlt-1, VEGF, and PlGF were measured on day 7 in several groups of animals (Figure 1D). Plasma sFlt-1 was increased in sFlt-1 transfection group. There was no significant change in plasma VEGF nor in PlGF among groups (Figure 1D).

Immunohistochemical staining indicated that compared with faint staining in the control artery, VEGF increased in the vicinity of inflammatory lesions (mononuclear cell infiltration) in the intima and adventitia on day 7 and in cells of 3 layers of cuffed artery on day 21 (Figure 2A). The endothelial layer, as detected by vWF staining, was preserved before and after cuff placement (Figure 2A). No detectable increase in PlGF staining was observed before and after cuff placement (Figure 2B).

Both Flk-1 and Flt-1 were undetectable, except in endothelial layers in control intact arteries, but both were increased in the intima, media, and adventitia 7 and 21 days after cuff placement (Figure 2A). Compared with Flt-1 staining, Flk-1 staining was less impressive on day 7 but was apparently noted on day 21. To localize VEGF receptors, immunofluorescent double-staining was performed (Figure 3). On day 7, α-SMA–positive cells in the media and neointima expressed little Flk-1, whereas they did express Flt-1 (Figure 3A). Also, some α-SMA–positive cells in the adventitia (possibly adventitial myofibroblasts) expressed Flt-1. Mac-3 positive cells recruited to the neointima, media, and adventitia expressed VEGF and Flt-1. On day 21, most α-SMA–positive cells in the neointima and media expressed VEGF and its receptors (Figure 3B).

Time Course of Development of Neointimal Hyperplasia in ApoE-KO Mice

As published,[24,27,31,32] within 7 days of cuff placement, mononuclear leukocytes, most of which were Mac3-positive monocytes, were recruited into the adventitia, media, and

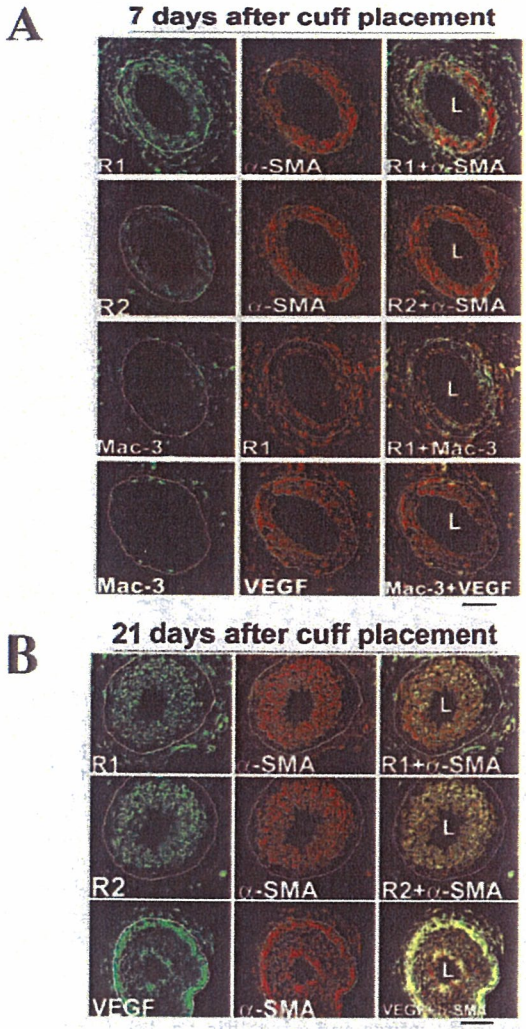

Figure 3. Immunofluorescence double-staining of VEGF receptors, monocytes, and α-SMA in cuffed femoral artery. A, Micrographs of cuffed femoral arteries doubly stained with Flt-1 (VEGF-R1, green) and α-SMA (red), with Flk-1 (VEGF-R2, green) and α-SMA (red), and with Mac-3 (green) and Flt-1 (VEGF-R1, red) 7 days after cuff placement. Scale bar=50 μm. B, Micrographs of cuffed femoral arteries doubly stained with Flt-1 (VEGF-R1) and α-SMA, Flk-1 (VEGF-R2) and α-SMA, and VEGF and α-SMA in the cuffed femoral arteries 21 days after cuff placement. Single fluorescence–positive cells were stained green or red, whereas double-positive cells were stained yellow. White lines indicate external elastin laminas. L indicates lumen. Scale bar=50 μm.

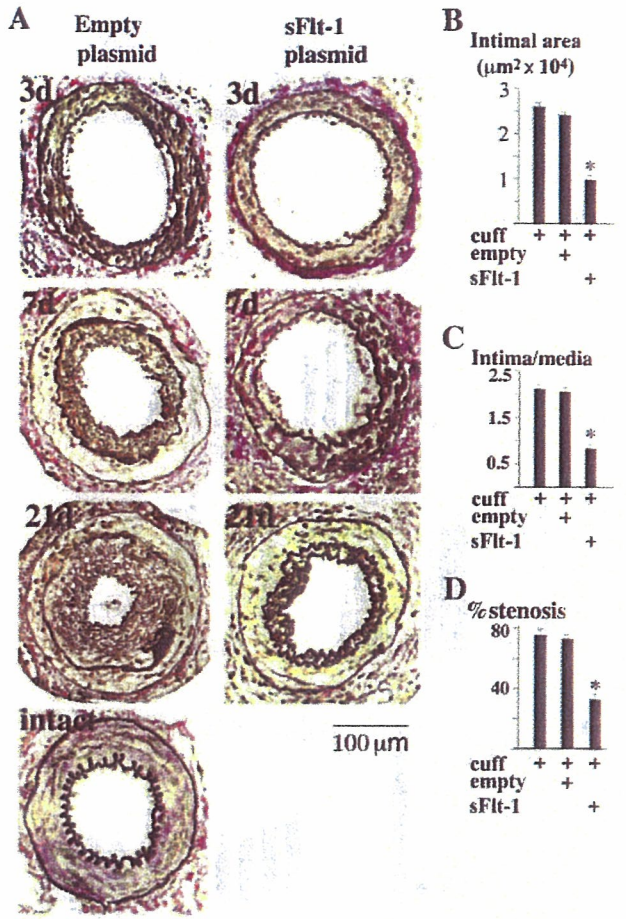

Figure 4. Histopathology of cuffed femoral artery. A, Time course of cuff injury–induced neointimal formation and effect of sFlt-1 gene transfer. Micrographs of cross sections of control (intact) and cuffed arteries stained with van Gieson Elastica on days 3, 7, and 21 are shown. Scale bar=100 μm. B through D, Effects of sFlt-1 gene transfer on neointimal thickening (B), intima/media ratio (C), and % stenosis (D) 21 days after cuff placement. *$P<0.01$ vs cuff only and cuff+empty plasmid group.

intima (Figure 3). After 7 days, neointimal lesions developed and became thick over time (Figure 4A). Monocyte infiltration declined spontaneously and α-SMA–positive cells appeared predominantly in the neointima. On day 21, significant neointimal formation with luminal stenosis developed (Figure 4A). Endothelial staining with vWF antibody showed that no significant neointimal neovascularization was observed during the course of experiments (Figure 2A).

Effects of Soluble Flt-1 Gene Transfer on Cuff-Induced Neointimal Hyperplasia in ApoE-KO Mice

As we previously reported,[27] Mac3-positive monocytes/macrophages and PCNA-positive cells were detected mainly in the adventitia and intima. There was markedly less inflammation (Mac3-positive cells) and proliferation (the PCNA index) in sFlt-1–transfected mice than in empty plasmid-transfected mice on day 7 (Figure II, available online at http://atvb.ahajournals.org). There was no detectable change in the number of CD3-positive T cells in empty plasmid or sFlt-1–transferred mice (Figure II). The sFlt-1 gene transfer significantly reduced neointimal formation (increases in neointimal area, intima/media ratio, and luminal stenosis) 21 days after cuff placement (Figure 4A through 4D).

Because sFlt-1 gene transfer markedly reduced monocyte-mediated inflammation, gene expression of a battery of inflammatory cytokines, chemokines, and chemokine receptors was examined by RNAse protection assays (Figure III, available online at http://atvb.ahajournals.org) or by RT-PCR (Figure 1C) 7 days after cuff placement. The sFlt-1 gene transfer did not affect gene expression of RANTES, macrophage inflammatory protein-1α, transforming growth factor-β, macrophage inflammatory protein-2, and PlGF, but prevented or attenuated the increased gene expression of CCR1, interleukin-6, CCR2, MCP-1, Flt-1, CXCR2, eotaxin,

vascular cell adhesion molecule-1, intercellular adhesion molecule-1, Flk-1, and VEGF. The sFlt-1 gene transfer reduced increased immunostainings against VEGF, Flt-1, and Flk-1, but did not affect staining against vWF (Figure 2A).

Time course of plasma concentrations of sFlt-1 after sFlt-1 gene transfer was determined. Plasma sFlt-1 levels before and 3, 7, and 14 days after sFlt-1 transfection were 467±37, 1037±132 ($P<0.01$ versus baseline), 927±215 ($P<0.01$), and 649±83 pg/mL ($P<0.05$, n=6 each), indicating that sFlt-1 was released from the transfected muscle.

Plasma Lipid Levels in ApoE-KO Mice

Total cholesterol and triacylglycerol levels were 503±11 and 38±6 mg/dL in the control group, 512±16 and 40±5 mg/dL in the empty plasmid group, and 497±10 and 39±3 mg/dL in the sFlt-1 group, indicating that the observed effects of sFlt-1 gene transfer were not caused by changes in serum lipid levels.

Effects of Flt-1 Tyrosine Kinase Deficiency on Neointimal Hyperplasia

Wild-type and Flt-1 tyrosine kinase–deficient mice were fed a high-fat diet for 2 weeks, and cuff was placed as mentioned above. Mice received a high-fat diet for an additional 3 weeks. Neointimal formation was noted 21 days after cuff placement in wild-type mice. Compared with wild-type mice, Flt-1 tyrosine kinase–deficient mice displayed reduced neointimal formation (Figure IV, available online at http://atvb.ahajournals.org). Total cholesterol levels at 5 weeks of high-fat diet were 520±21 and 511±18 mg/dL in wild-type and Flt-1 tyrosine kinase–deficient mice, respectively.

Discussion

This study is the first to demonstrate the essential role of VEGF and Flt-1 signals in the development of neointimal formation after cuff-induced periadventitial injury in hypercholesterolemic mice. VEGF is conventionally thought to be an endothelial cell–specific growth factor and to attenuate vascular disease by inducing endothelial proliferation and regeneration mainly through the endothelial type 2 receptor Flk-1.[7] Recent evidence, however, suggests that functional Flt-1 and Flk-1 are expressed in injured arterial wall cells other than endothelial cells. In this study, Flt-1, Flk-1, and VEGF were increased in lesional monocytes and medial smooth muscle cells at early stages and in neointimal and medial smooth muscle cells at later stages. Flt-1 is demonstrated to act as an important mediator of chemotaxis through vascular cell adhesion molecule-1, intercellular adhesion molecule-1, and MCP-1.[13–15] We demonstrated that sFlt-1 gene transfer reduced the early inflammatory and proliferative changes and thus attenuated the development of neointimal formation. It is speculated, therefore, that VEGF might cause inflammation (monocyte recruitment and activation) and proliferation through Flt-1–mediated signals and thus cause neointimal formation after cuff-induced periadventitial injury. In addition, Flt-1 in smooth muscle cells is reported to mediate migration and proliferation in vitro.[18,33] An alternative interpretation is that VEGF directly caused migration and proliferation of smooth muscle cells resulting in neointimal formation. In any way, our present finding of Flt-1 tyrosine kinase–deficient mice suggests the involvement of Flt-1–related signals in the pathogenesis of neointimal formation after periadventitial injury.

Periadventitial inflammation has a major role in the pathogenesis of cuff-induced neointimal formation.[27,32] To gain insight into the mechanism of VEGF-mediated inflammation and neointimal formation, we assessed gene expression of various inflammatory genes. The sFlt-1 gene transfer attenuated increased gene expression of inflammatory cytokines, adhesion molecules, chemokines, and chemokine receptors. Yamada et al[34] showed that MCP-1 is essential in VEGF-induced angiogenesis, vascular leakage, and inflammation. An essential role of MCP-1 in the development of neointimal formation after arterial injury has also been reported.[27–29,35,36] The sFlt-1 gene transfer attenuated increased VEGF, Flk-1, and Flt-1 gene expression, indicating that VEGF regulates its activity by an autocrine loop mechanism within diseased arterial wall cells, such as smooth muscle cells, endothelial cells, and lesional monocytes. A positive feedback effect of VEGF is supported by previous studies that demonstrated enhanced VEGF production by monocytes through Flt-1 stimulation.[37] Therefore, sFlt-1 gene transfer might attenuate cuff-induced neointimal formation mainly by suppressing inflammation (monocyte recruitment and activation).

This study has potentially significant clinical implications. Blockade of VEGF by sFlt-1 gene transfer can be an attractive anti-VEGF therapy for inflammatory vascular disease and other inflammatory disorders. The efficacy of this strategy for experimental tumor angiogenesis has already been tested.[22] Luttun et al[38] recently reported that treatment with anti–Flt-1 antibody attenuated the development of experimental tumor angiogenesis, arthritis, and atherosclerosis. One limitation of the present finding is that the pathogenesis of neointimal formation after periadventitial injury differs from that after endothelial injury/denudation and from that in humans. The endothelial integrity is preserved in cuff-induced periadventitial injury. For clinical application of our findings to human vascular disease, future studies are needed to examine the efficacy and safety of anti-VEGF strategy with sFlt-1 in experimental atherosclerosis and restenosis.

In conclusion, inflammatory changes mediated by VEGF and Flt-1 signals play an important role in the pathogenesis of neointimal formation after cuff-induced periadventitial injury. These data support the notion that VEGF promote neointimal formation by acting as a proinflammatory cytokine after cuff-induced periadventitial injury.

Acknowledgments

This study was supported by Grants-in-Aid for Scientific Research (14657172, 14207036) from the Ministry of Education, Science, and Culture by Health Science Research Grants (Comprehensive Research on Aging and Health and Research on Translational Research), from the Ministry of Health, Labor, and Welfare, and by the Program for Promotion of Fundamental Studies in Health Sciences of the Organization for Pharmaceutical Safety and Research, Tokyo, Japan.

References

1. Libby P, Ganz P. Restenosis revisited: new targets, new therapies. *N Engl J Med.* 1997;337:418–419.

2. Topol EJ, Serruys PW. Frontiers in interventional cardiology. *Circulation*. 1998;98:1802–1820.
3. Shibata M, Suzuki H, Nakatani M, Koba S, Geshi E, Katagiri T, Takeyama Y. The involvement of vascular endothelial growth factor and flt-1 in the process of neointimal proliferation in pig coronary arteries following stent implantation. *Histochem Cell Biol*. 2001;116:471–481.
4. Ruef J, Hu ZY, Yin LY, Wu Y, Hanson SR, Kelly AB, Harker LA, Rao GN, Runge MS, Patterson C. Induction of vascular endothelial growth factor in balloon-injured baboon arteries. A novel role for reactive oxygen species in atherosclerosis. *Circ Res*. 1997;81:24–33.
5. Chen YX, Nakashima Y, Tanaka K, Shiraishi S, Nakagawa K, Sueishi K. Immunohistochemical expression of vascular endothelial growth factor/vascular permeability factor in atherosclerotic intimas of human coronary arteries. *Arterioscler Thromb Vasc Biol*. 1999;19:131–139.
6. Inoue M, Itoh H, Ueda M, Naruko T, Kojima A, Komatsu R, Doi K, Ogawa Y, Tamura N, Takaya K, Igaki T, Yamashita J, Chun TH, Masatsugu K, Becker AE, Nakao K. Vascular endothelial growth factor (VEGF) expression in human coronary atherosclerotic lesions: possible pathophysiological significance of VEGF in progression of atherosclerosis. *Circulation*. 1998;98:2108–2116.
7. Baumgartner I, Isner JM. Somatic gene therapy in the cardiovascular system. *Annu Rev Physiol*. 2001;63:427–450.
8. Zachary I, Mathur A, Yla-Herttuala S, Martin J. Vascular protection: a novel nonangiogenic cardiovascular role for vascular endothelial growth factor. *Arterioscler Thromb Vasc Biol*. 2000;20:1512–1520.
9. Zachary I, Gliki G. Signaling transduction mechanisms mediating biological actions of the vascular endothelial growth factor family. *Cardiovasc Res*. 2001;49:568–581.
10. Bussolati B, Ahmed A, Pemberton H, Landis RC, Di Carlo F, Haskard DO, Mason JC. Bifunctional role for VEGF-induced heme oxygenase-1 in vivo: induction of angiogenesis and inhibition of leukocytic infiltration. *Blood*. 2004;103:761–766.
11. Isner JM. Still more debate over VEGF. *Nat Med*. 2001;7:639–641.
12. Ware JA. Too many vessels? Not enough? The wrong kind? The VEGF debate continues. *Nat Med*. 2001;7:403–404.
13. Barleon B, Sozzani S, Zhou D, Weich HA, Mantovani A, Marme D. Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1. *Blood*. 1996;87:3336–3343.
14. Kim I, Moon SO, Kim SH, Kim HJ, Koh YS, Koh GY. Vascular endothelial growth factor expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin through nuclear factor-kappa B activation in endothelial cells. *J Biol Chem*. 2001;276:7614–7620.
15. Marumo T, Schini-Kerth VB, Busse R. Vascular endothelial growth factor activates nuclear factor-kappaB and induces monocyte chemoattractant protein-1 in bovine retinal endothelial cells. *Diabetes*. 1999;48:1131–1137.
16. Celletti FL, Waugh JM, Amabile PG, Brendolan A, Hilfiker PR, Dake MD. Vascular endothelial growth factor enhances atherosclerotic plaque progression. *Nat Med*. 2001;7:425–429.
17. Grosskreutz CL, Anand-Apte B, Duplaa C, Quinn TP, Terman BI, Zetter B, D'Amore PA. Vascular endothelial growth factor-induced migration of vascular smooth muscle cells in vitro. *Microvasc Res*. 1999;58:128–136.
18. Ishida A, Murray J, Saito Y, Kanthou C, Benzakour O, Shibuya M, Wijelath ES. Expression of vascular endothelial growth factor receptors in smooth muscle cells. *J Cell Physiol*. 2001;188:359–368.
19. Moulton KS, Heller E, Konerding MA, Flynn E, Palinski W, Folkman J. Angiogenesis inhibitors endostatin or TNP-470 reduce intimal neovascularization and plaque growth in apolipoprotein E-deficient mice. *Circulation*. 1999;99:1726–1732.
20. Kendall RL, Wang G, Thomas KA. Identification of a natural soluble form of the vascular endothelial growth factor receptor, FLT-1, and its heterodimerization with KDR. *Biochem Biophys Res Commun*. 1996;226:324–328.
21. Zhao Q, Egashira K, Inoue S, Usui M, Kitamoto S, Ni W, Ishibashi M, Hiasa Ki K, Ichiki T, Shibuya M, Takeshita A. Vascular endothelial growth factor is necessary in the development of arteriosclerosis by recruiting/activating monocytes in a rat model of long-term inhibition of nitric oxide synthesis. *Circulation*. 2002;105:1110–1115.
22. Goldman CK, Kendall RL, Cabrera G, Soroceanu L, Heike Y, Gillespie GY, Siegal GP, Mao X, Bett AJ, Huckle WR, Thomas KA, Curiel DT. Paracrine expression of a native soluble vascular endothelial growth factor receptor inhibits tumor growth, metastasis, and mortality rate. *Proc Natl Acad Sci U S A*. 1998;95:8795–8800.
23. Hiratsuka S, Minowa O, Kuno J, Noda T, Shibuya M. Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. *Proc Natl Acad Sci U S A*. 1998;95:9349–9354.
24. Lardenoye JH, Delsing DJ, de Vries MR, Deckers MM, Princen HM, Havekes LM, van Hinsbergh VW, van Bockel JH, Quax PH. Accelerated atherosclerosis by placement of a perivascular cuff and a cholesterol-rich diet in ApoE*3Leiden transgenic mice. *Circ Res*. 2000;87:248–253.
25. von der Thusen JH, van Berkel TJ, Biessen EA. Induction of rapid atherogenesis by perivascular carotid collar placement in apolipoprotein E-deficient and low-density lipoprotein receptor-deficient mice. *Circulation*. 2001;103:1164–1170.
26. Kondo K, Hiratsuka S, Subbalakshmi E, Matsushime H, Shibuya M. Genomic organization of the flt-1 gene encoding for vascular endothelial growth factor (VEGF) receptor-1 suggests an intimate evolutionary relationship between the 7-Ig and the 5-Ig tyrosine kinase receptors. *Gene*. 1998;208:297–305.
27. Egashira K, Zhao Q, Kataoka C, Ohtani K, Usui M, Charo IF, Nishida K, Inoue S, Katoh M, Ichiki T, Takeshita A. Importance of monocyte chemoattractant protein-1 pathway in neointimal hyperplasia after periarterial injury in mice and monkeys. *Circ Res*. 2002;90:1167–1172.
28. Usui M, Egashira K, Ohtani K, Kataoka C, Ishibashi M, Hiasa K, Katoh M, Zhao Q, Kitamoto S, Takeshita A. Anti-monocyte chemoattractant protein-1 gene therapy inhibits restenotic changes (neointimal hyperplasia) after balloon injury in rats and monkeys. *FASEB J*. 2002;16:1838–1840.
29. Mori E, Komori K, Yamaoka T, Tanii M, Kataoka C, Takeshita A, Usui M, Egashira K, Sugimachi K. Essential role of monocyte chemoattractant protein-1 in development of restenotic changes (neointimal hyperplasia and constrictive remodeling) after balloon angioplasty in hypercholesterolemic rabbits. *Circulation*. 2002;105:2905–2910.
30. Aihara H, Miyazaki J. Gene transfer into muscle by electroporation in vivo. *Nat Biotechnol*. 1998;16:867–870.
31. Moroi M, Zhang L, Yasuda T, Virmani R, Gold HK, Fishman MC, Huang PL. Interaction of genetic deficiency of endothelial nitric oxide, gender, and pregnancy in vascular response to injury in mice. *J Clin Invest*. 1998;101:1225–1232.
32. Wu L, Iwai M, Nakagami H, Li Z, Chen R, Suzuki J, Akishita M, de Gasparo M, Horiuchi M. Roles of angiotensin II type 2 receptor stimulation associated with selective angiotensin II type 1 receptor blockade with valsartan in the improvement of inflammation-induced vascular injury. *Circulation*. 2001;104:2716–2721.
33. Wang H, Keiser JA. Vascular endothelial growth factor upregulates the expression of matrix metalloproteinases in vascular smooth muscle cells: role of flt-1. *Circ Res*. 1998;83:832–840.
34. Yamada M, Kim S, Egashira K, Takeya M, Ikeda T, Mimura O, Iwao H. Molecular mechanism and role of endothelial monocyte chemoattractant protein-1 induction by vascular endothelial growth factor. *Arterioscler Thromb Vasc Biol*. 2003;23:1996–2001.
35. Egashira K. Molecular mechanisms mediating inflammation in vascular disease: special reference to monocyte chemoattractant protein-1. *Hypertension*. 2003;41:834–841.
36. Ohtani K, Usui M, Nakano K, Kohjimoto Y, Kitajima S, Hirouchi Y, Li XH, Kitamoto S, Takeshita A, Egashira K. Antimonocyte chemoattractant protein-1 gene therapy reduces experimental in-stent restenosis in hypercholesterolemic rabbits and monkeys. *Gene Ther*. 2004;11:1273–1282.
37. Bottomley MJ, Webb NJ, Watson CJ, Holt L, Bukhari M, Denton J, Freemont AJ, Brenchley PE. Placenta growth factor (PlGF) induces vascular endothelial growth factor (VEGF) secretion from mononuclear cells and is co-expressed with VEGF in synovial fluid. *Clin Exp Immunol*. 2000;119:182–188.
38. Luttun A, Tjwa M, Moons L, Wu Y, Angelillo-Scherrer A, Liao F, Nagy JA, Hooper A, Priller J, De Klerck B, Compernolle V, Daci E, Bohlen P, Dewerchin M, Herbert JM, Fava R, Matthys P, Carmeliet G, Collen D, Dvorak HF, Hicklin DJ, Carmeliet P. Revascularization of ischemic tissues by PlGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1. *Nat Med*. 2002;8:831–840.

Critical Role for Monocyte Chemoattractant Protein-1 and Macrophage Inflammatory Protein-1α in Induction of Experimental Autoimmune Myocarditis and Effective Anti–Monocyte Chemoattractant Protein-1 Gene Therapy

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Background—Autoimmune myocarditis is a principal cause of heart failure among young adults and is often a precursor of dilated cardiomyopathy. Monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1α (MIP-1α) are potent chemotactic factors for mononuclear cells. The inflammatory infiltrate observed in myocardial lesions of myocarditis consists of >70% mononuclear cells. To determine their critical role in the pathogenesis of myocarditis, we inhibited mononuclear cell activation and migration to see if it would affect disease severity and disease prevalence in experimental autoimmune myocarditis (EAM).

Methods and Results—In this report, we demonstrated that blockade of MCP-1 or MIP-1α with monoclonal antibodies significantly reduced severity of myocarditis in BALB/c mice immunized with cardiac myosin. Similar results were obtained when CCR2−/− and CCR5−/− mice were used. In CCR2−/− mice, not only disease severity but also disease prevalence was reduced. To further inhibit mononuclear cell activation and migration, we transfected the mice before inducing EAM with a dominant-negative inhibitor of MCP-1 gene (7ND). This transfection significantly reduced the disease severity, decreased mRNA expression levels, especially of the chemokines RANTES, MIP-2, IP-10, MCP-1, T-cell activation gene 3, and eotaxin in the myocardium, and resulted in a reduction in cardiac myosin-induced interleukin-1 and interleukin-4 and in an increase in interferon-γ and interleukin-10 cytokine production by splenocytes.

Conclusions—Overall, these findings suggest that the chemokines MCP-1 and MIP-1α, acting through their receptors CCR2 and CCR5, are important in the induction of EAM and that inhibition of MCP-1 with 7ND gene transfection significantly reduced disease severity. This strategy may be a new feasible form of gene therapy against autoimmune myocarditis. (Circulation. 2005;112:3400-3407.)

Key Words: myosin ■ myocarditis ■ inflammation ■ gene therapy

The attraction of leukocytes from blood to tissue is essential for inflammation and the host response to infection. This process is partly controlled by chemokines and their receptors. Monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1α (MIP-1α) are members of the C-C chemokine family, which has been shown to play a major role in the migration of monocytes to an inflammatory focus.[1,2] MCP-1 can also attract memory T cells and activated natural killer cells in vitro[3] and is key to the development of Th2 responses.[4] CCR2, the receptor for MCP-1, is highly expressed on monocytes and activated T cells.[5] Similar to MCP-1, MIP-1α can mediate the recruitment of monocytes in several inflammatory diseases.[6] CCR5, the major receptor for MIP-1α, is expressed mostly on activated T cells and monocytes/macrophages.[5]

Inflammation of the heart muscle (myocarditis) is a frequent cause of cardiac failure in young adults. Autoimmunity plays an important role in human myocarditis and contributes to the progression to cardiomyopathy and heart failure.[6] Fuse and colleagues[7] recently showed increased mRNA levels of MCP-1 in the hearts of rats immunized with cardiac myosin. Also, serum MCP-1 levels of the rats with experimental autoimmune myocarditis (EAM) were significantly elevated from days 15 until 24. In the clinical study, they further showed that serum levels of MCP-1 in patients with acute myocarditis were significantly elevated compared with those

Received June 29, 2005; revision received August 12, 2005; accepted September 2, 2005.

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The online-only Data Supplement, which contains Figure I and Figure II, can be found with this article at http://circ.ahajournals.org/cgi/content/full/112/22/3400/DC1.

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Circulation is available at http://www.circulationaha.org

DOI: 10.1161/CIRCULATIONAHA.105.572396

of healthy volunteers. However, the precise role of this chemokine in the pathogenesis of myocarditis remains undetermined. To further clarify the role of chemokines such as MCP-1 and MIP-1α and their receptors CCR2 and CCR5 in the pathogenesis of myocarditis and to establish new therapeutic approaches, we first blocked in BALB/c mice immunized with cardiac myosin MCP-1 or MIP-1α by using monoclonal antibodies. We then used CCR2−/− or CCR5−/− mice to further analyze the role of these chemokines and their receptors. Finally, we studied the use of gene therapy to block MCP-1 activity in vivo by using an N-terminal deletion mutant of MCP-1, called 7ND, which lacks the N-terminal amino acids 2 to 8. This mutant MCP-1 has been shown to bind the receptor for MCP-1, CCR2, and block MCP-1–mediated monocyte chemotaxis.[8,9] Because the inflammatory infiltrate observed in myocardial lesions of myocarditis consists of >70% mononuclear cells,[10] these approaches may also open novel therapeutic strategies to treat myocarditis in humans.

Methods

Mice

Female BALB/c mice (6 to 8 weeks of age) were obtained from Charles River Laboratories (Sulzfeld, Germany) and maintained in the conventional animal facility at the University of Heidelberg, and these mice were used in all experiments except the experiments done with CCR2−/− and CCR5−/− mice. CCR2−/− and CCR5−/− mice (both hybrids of C57B1/6 and 129/C57B1/6 respectively, and kindly provided by W.A. Kuziel, University of Texas, Austin) were back-crossed for 6 generations onto the BALB/c background. The CCR2−/+ and CCR5−/+ mice were then interbred to generate CCR2+/+ and CCR5+/+ mice on the same genetic background. The animal work was approved by the Animal Care and Use Committee of the University of Heidelberg.

Antigen Preparation and Induction of Myocarditis

Murine cardiac myosin was purified from pooled mouse hearts through the use of a previously described procedure.[11,12] The purified cardiac myosin was emulsified with equal volume of complete Freund's adjuvant (Sigma) containing 5 mg/mL of Mycobacterium tuberculosis H37Ra (Sigma). Each mouse was injected subcutaneously with 100 μL of emulsion containing 200 μg of cardiac myosin on days 0 and 7.

Histopathological Evaluation

For the histopathological evaluation, mice were euthanized on day 21, and serial sections were made through the heart. Every fifth section was stained with hematoxylin and eosin. Evidence of myocarditis was evaluated with light microscopy independently and in a blinded manner by 2 pathologists according to a 6-tier scoring system: Grade 0, no inflammation; grade 1, cardiac infiltration in up to 5% of the cardiac sections; grade 2, 6% to 10%; grade 3, 11% to 30%; grade 4, 31% to 50%; and grade 5, >50%. The average score from the pathologist's readings was taken for statistical analysis with a nonparametric test.

MCP-1 and MIP-1α Blockade

The rat anti-mouse MCP-1 monoclonal antibody (CCL2/JE/MCP-1 mAb, R&D Systems) was used to block MCP-1, and rat anti-mouse MIP-1α mAb (CCL3/MIP-1α mAb, R&D Systems) was used to block MIP-1α. Rat IgG2b mAb from clone A95–1 (PharMingen) was used as an isotype-matched control. BALB/c mice were injected intraperitoneally with MCP-1 mAbs or MIP-1α or isotype-matched control monoclonal antibodies (500 μg of either) in phosphate-buffered saline (PBS; 0.1 mL) on days 12 and 17. These injection times were selected because Fuse and colleagues[7] recently showed that serum MCP-1 levels in rats with EAM were significantly elevated from days 15 until 24.

PCR Genotyping

CCR2 Mice

To detect CCR2 knockout alleles, the primer pair of WAK121 (5′-TTCCATTGCTCAGCGGTGCT-3′) and WAK134 (5′-TCAGAGATGGCCAAGTTGAGC-AGA-3′) yield a polymerase chain reaction product of 450 bp.

CCR5 Mice

To detect CCR5 knockout alleles, the primer pair of WAK121 (5′-TTCCATTGCTCAGCGGTGCT-3′) and WAK131 (5′-TGTTTCCTCCTCTAGCCTTCAC-TATG-3′) yield a polymerase chain reaction product of 350 bp.

Plasmid Expression Vectors

Human 7ND complementary deoxyribonucleic acid (cDNA) was constructed by a recombinant chain reaction using wild-type human MCP-1 cDNA (generous gift from T. Yoshimura, National Cancer Institute, Bethesda, Md) as a template and inserted into the BamHI (5′) and NotI (3′) sites of the pcDNA3 expression vector plasmid (Invitrogen) as described previously.[13] All sequences were confirmed by sequencing.

Gene Transfer

Gene transfer was done as described before.[14] The mice were injected either with 7ND gene or with control plasmid (50 μg in 30 μL PBS) or with 30 μL PBS alone in the femoral muscle using a 27-gauge needle under anesthesia by intraperitoneal injection of pentobarbital. To enhance transgene expression, all mice received electroporation at the injected site.

Detection of Serum Concentrations of Anti–Cardiac Myosin Titers, Cardiac Myosin–Specific Cytokine Production by Splenocytes

The detection of serum concentrations of anti-cardiac myosin titers and cardiac myosin-specific cytokine production by splenocytes was done as previously described.[11,12] To detect serum anti–cardiac myosin titers, plates were coated with 100 μL/well of cardiac myosin (5 μg/mL) in bicarbonate buffer (pH 9.6) and left overnight. Mouse secondary antibodies, diluted to 1:1000 for IgG (KPL), IgG1, IgG2a (PharMingen), and IgG2b (Bethyl), were used for detection. Serum samples from test mice were diluted to 1:50, 1:200, 1:800, 1:3200, and 1:12800. Normal mouse serum was used as control. Optical densities were determined at 450 nm. Antibody end point titers for each individual mouse were calculated as the greatest positive dilution of antibody.

For in vitro cytokine production, the splenocytes were cultured at 5×10^6 per well in Roswell Park Memorial Institute 1640 complete medium in the presence of 30 μg/mL of cardiac myosin or medium alone for 48 hours. Supernatant was collected, aliquoted, and frozen at −20°C. Cytokines were measured by Quantikine cytokine ELISA kits (R&D Systems) according to the manufacturer's instructions.

RNA Protection Assay

The mCR-5 cytokine receptor multiprobe template set (BD Biosciences Pharmingen) was used to measure mouse mRNAs encoding CCR1, CCR2, CCR1b, CCR3, CCR4, and CCR5. The mCK-5c multiprobe template set (BD Biosciences Pharmingen) was used to measure mouse mRNAs encoding Ltn, RANTES, MIP-1b, MIP-1a, MIP-2, IP-10, MCP-1, T-cell activation gene 3 (TCA-3), and eotaxin. The measurements were performed according to the manufacturer's guidelines.

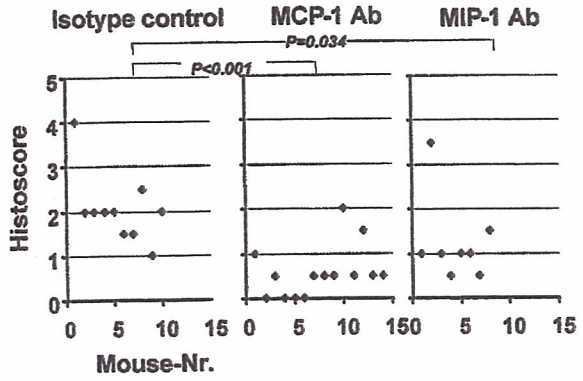

Figure 1. Blocking MCP-1 and MIP-1α. Effects on the severity and prevalence of myocarditis.

Statistical Analysis

Normally distributed data were analyzed by Student's t test; otherwise, the Mann-Whitney U test was used. Disease prevalence was compared by using a χ^2 2-way analysis. Statistical comparison across 3 groups were calculated with the use of ANOVA followed by appropriately conducted multiple comparisons. Probability values of <0.05 were considered significant.

Results

Blocking MCP-1 With Monoclonal Antibodies Reduced the Severity of Disease in EAM

To determine the effect of blocking MCP-1, we induced EAM in BALB/c mice by immunization with cardiac myosin and treated the mice either with MCP-1 mAbs or with the isotype control on days 12 and 17. Compared with the isotype controls, mice treated with MCP-1 mAbs showed a significant decrease in the prevalence and severity of myocarditis (Figure 1). Only 3 of 14 mice treated with MCP-1 mAbs developed myocarditis with lesion grade of 1 and more, whereas no or very little inflammation was detected in the rest of the group. In contrast, all isotype-treated control mice had myocarditis with a lesion grade of 1 and more.

Blocking MIP-1α With Monoclonal Antibodies Reduced the Severity of Disease in EAM

To further study the effect of blocking MIP-1α, EAM was induced in BALB/c mice by immunization with cardiac myosin. They were treated either with MIP-1α mAbs or with the isotype control on days 12 and 17. Compared with the isotype controls, mice treated with MIP-1α mAbs showed a significant decrease in the prevalence and severity of myocarditis (Figure 1). Compared with mice treated with MCP-1 mAbs, the decrease was less but still significant. Four of 8 mice had myocarditis with grade 1 lesions and only 2 of 8 mice treated with MIP-1α mAbs had myocarditis with lesion grade 1.5 and more. In contrast, 9 of 10 mice treated with the isotype-matched control mAb had myocarditis with a lesion grade of 1.5 and more.

CCR2−/− and CCR5−/− Mice Show a Reduced Severity of EAM

To examine the role of CCR2, the major receptor for MCP-1, and the role of CCR5, the main receptor for MIP-1α, in the pathogenesis of EAM, we immunized CCR2−/−, CCR2+/+, CCR5−/−, and CCR5+/+ mice with cardiac myosin. Compared with the CCR2+/+ mice, the CCR2−/− mice showed a significant reduction in severity of myocarditis (Figure 2). Only 1 of 14 CCR2−/− mice had myocarditis with a grade 1 and more, whereas 10 of 15 CCR2+/+ mice had myocarditis with grade 1 or greater severity (Figure 3, a through f). The prevalence of EAM was also significantly lower in CCR2−/− mice (33% versus 86%) (Figure 2). Furthermore, interleukin (IL)-1 and IL-4 production by splenocytes was significantly reduced, whereas interferon-γ (IFN-γ) and IL-10 production was increased in CCR2−/− mice (Figure 4).

Similar results were obtained when CCR5−/− mice were used. CCR5−/− mice showed a significant decrease in disease severity. Two of 12 CCR5−/− mice had myocarditis with grade 1 or greater, whereas 8 of 11 CCR5+/+ mice had myocarditis with grade 1 or greater (Figure 2). TNF-α production by splenocytes was significantly reduced in CCR5−/− mice, whereas IL-10 production was increased (Figure 4).

There were no significant differences in autoantibody production (tested for following subclasses of IgG: IgG1, IgG2a, and IgG2b) against cardiac myosin in both groups of mice.

mRNA Expression of Chemokines and Chemokine Receptors in the Myocardium

To look at the expression of different chemokines and chemokine receptors, we measured the mRNA expression levels of the chemokines Ltn, RANTES, MIP-1b, MIP-1a, MIP-2, interferon-inducible protein (IP)-10, MCP-1, TCA-3,

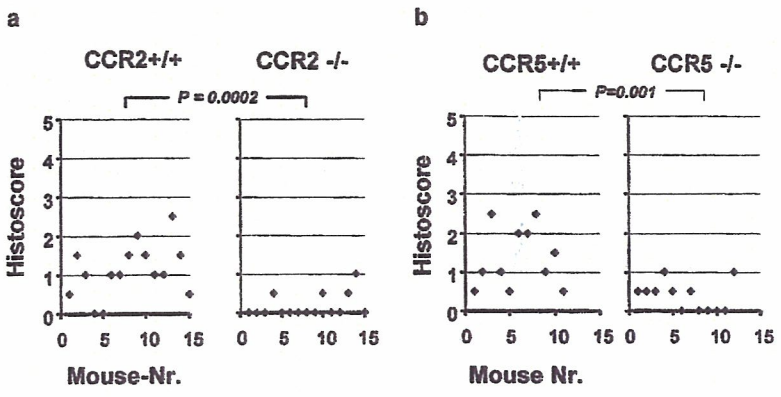

Figure 2. Histoscore. Immunization of CCR2+/+ and CCR2−/− mice (A) and CCR5+/+ and CCR5−/− mice (B) with cardiac myosin. Effect on the severity and prevalence of myocarditis is shown.

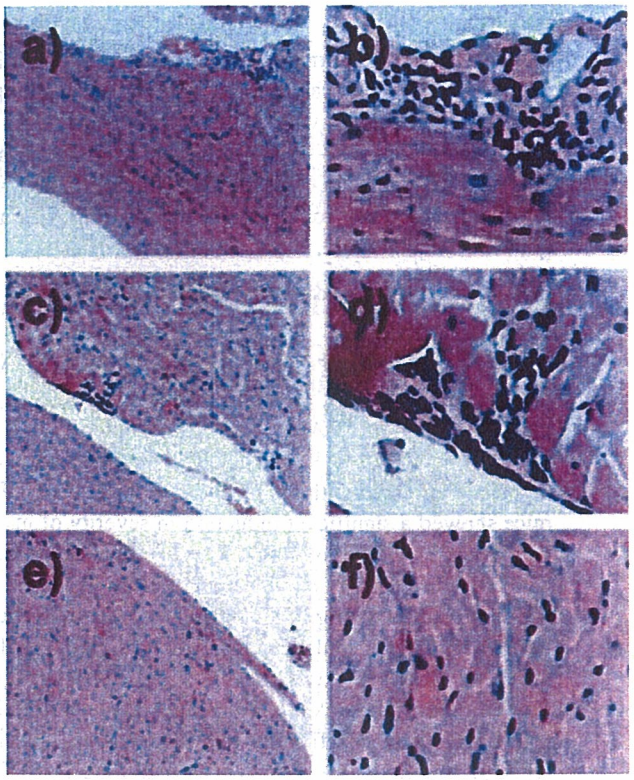

Figure 3. Histological examination of hearts of CCR2+/+ and CCR2−/− mice immunized with cardiac myosin. Hearts were removed on day 21 for histological examination. a, Original magnification ×16; b, ×40. Heart infiltrate that was observed in one CCR2+/+ mouse immunized with cardiac myosin (grade 2.5). c, Original magnification ×16; d, ×40. Heart with grade 1 lesion represents the only mouse out of 15 mice that had the most severe myocarditis in the CCR2−/− group of mice. e, Original magnification ×16; f, ×40. Representative heart section of the 13 mice (2 in the CCR2+/+ and 11 in the CCR2−/− group of mice) that did not have any infiltration.

and eotaxin, and of the chemokine receptors CCR1, CCR2, CCR1b, CCR3, CCR4, and CCR5 in the myocardium of these mice. We were able to detect mRNA levels for all chemokines tested (Figure I), but the expression of mRNA levels was markedly higher for the chemokines RANTES, MIP-2, IP-10, and MCP-1 in all mice. The mRNA expression levels of RANTES, MIP-2, IP-10, and MCP-1 correlated with the severity of myocarditis. Thus, all mice with lower myocarditis scores showed lower expression of these chemokines compared with mice with higher myocarditis independent of being CCR2- or CCR5-deficient or wild-type mice. Further, we found that primarily the mRNA for the CCR receptors CCR1, CCR2, and CCR5 was expressed in the myocardium of all tested mice (Figure I). There was no mRNA or very low levels for the chemokine receptors CCR1b, CCR3, and CCR4. CCR2−/− and CCR5−/− mice showed lower mRNA expression of the chemokine receptors CCR1 and CCR5 (for CCR2−/− mice) and CCR1 and CCR2 (for CCR5−/− mice) compared with the CCR2+/+ and CCR5+/+ mice. There was no compensatory upregulation of other chemokine receptors when CCR2 receptor or CCR5 receptor knockout mice were used. Similar to the chemokines, the upregulation of mRNA levels of these chemokine receptors were correlated with the severity of inflammation. Mice with lower myocarditis scores showed greater reduction of mRNA of these chemokine receptors compared with mice with higher myocarditis score (data not shown).

7ND Gene Transfection Decreased the Inflammation in EAM

We determined the effect of 7ND gene transfection and examined whether anti–MCP-1 gene therapy using the 7ND gene construct is effective. Compared with the control plasmid treatment group and PBS group, mice treated with the 7ND gene showed a marked decrease in the prevalence and severity of myocarditis, changes in cytokine profiles, and mRNA expression levels for chemokines (Figure 5, Figure 6, and Figure II). Only 1 of 11 mice treated with the 7ND gene had mild myocarditis with a lesion grade of 1 or more, whereas no or little inflammation was detected in the rest of the group. In contrast, 8 of 12 PBS-treated mice and 8 of 12 control plasmid-treated mice had myocarditis with lesion grades of 1 or more (Figure 5 and Figure II). Cardiac myosin–specific production of IL-1 and IL-4 (compared with control plasmid-treated and PBS-treated mice) by splenocytes were significantly reduced in mice treated with 7ND, whereas

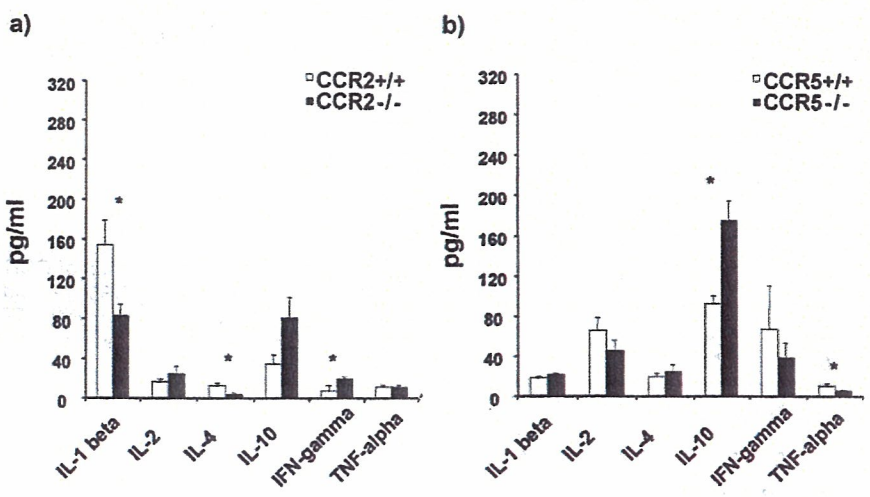

Figure 4. Immunization of CCR2+/+ and CCR2−/− mice (A) and CCR5+/+ and CCR5−/− mice (B) with cardiac myosin. Effect on cytokine production by splenocytes after cardiac myosin stimulation at day 21 is shown. *P<0.05.